Functionally selective inhibition of the oxytocin receptor by retosiban in human myometrial smooth muscle

Paul J Brighton[1], Michael J. Fossler[2], Siobhan Quenby[1,3], Andrew M Blanks[1,4]

1: Cell and Developmental Biology, Division of Biomedical Sciences, Warwick Medical School, University of Warwick. CV2 2DX

2: Senior Director, Clinical Pharmacology Modeling and Simulation, GlaxoSmithKline, Upper Merion West, King of Prussia, PA.

Current address: Vice President, Clinical Development and Quantitative Sciences, Trevena Inc., Chesterbrook, PA.

3: Maternity Directorate, University Hospital Coventry and Warwickshire, Coventry. CV2 2DX

4: **Address correspondence to:** Andrew M. Blanks, Cell and Developmental Biology, Division of Biomedical Sciences, Warwick Medical School, Coventry CV2 2DX, UK. P: +44-2476968703 F: +44-2476968653; Email:andrew.blanks@warwick.ac.uk

Acknowledgments:

We thank the patients and midwives of the Biomedical Research Unit at University Hospital Coventry and Warwickshire for the collection and curation of myometrial samples.

Funding:

This work was funded by a project grant to A.M.B, S.Q. and P.J.B. from GlaxoSmithKline

Conflict of interest:

A.M.B. has undertaken scientific consultancy work for GlaxoSmithKline and Ferring pharmaceuticals for the development of oxytocin receptor antagonists for the treatment of preterm birth.

Author Contributions:

A.M.B. conceived of the project, acquired the grant, analysed data and co-wrote the manuscript. P.J.B. undertook all experimental work, analysed data and co-wrote the manuscript. S.Q. acquired and curated patient samples and edited the manuscript. M.J.F. co-conceived the project, analysed data and edited the manuscript.

Abstract

Context: Novel small molecule inhibitors of the oxytocin receptor (OTR) may have distinct pharmacology and mode of action when compared to first generation oxytocin antagonists when used for the prevention of preterm birth.

Objective: To determine the mechanism of action of small molecule OTR antagonists retosiban and epelsiban in comparison to the currently used peptide-based compound atosiban.

Design: Human myometrial samples were obtained at cesarean section and subjected to pharmacological manipulations to establish the effect of antagonist binding to OTR on downstream signaling.

Results: Retosiban antagonism of oxytocin action in human myometrium was potent, rapid and reversible. Inhibition of inositol 1,4,5-trisphosphate (IP3) production followed single site competitive binding kinetics for epelsiban, retosiban and atosiban. Retosiban inhibited basal production of IP3 in the absence of oxytocin. Oxytocin and atosiban, but not retosiban inhibited forskolin and calcitonin stimulated cAMP production. Inhibition of cAMP was reversed by pertussis toxin. Oxytocin and atosiban, but not retosiban and epelsiban, stimulated ERK1/2 activity in a time a concentration dependent manner. Oxytocin and atosiban stimulated cyclo oxygenase 2 (COX2) activity and subsequent production of prostaglandin E_2 and $F_{2\alpha}$. Prostaglandin production was inhibited by rofecoxib, pertussin toxin, and ERK inhibitor U0126. Oxytocin but not retosiban or atosiban stimulated coupling of the OTR to $G\alpha_q$ G-proteins. Oxytocin and atosiban but not retosiban stimulated coupling of the OTR to $G\alpha_i$ G-proteins.

Conclusions:

Retosiban and epelsiban demonstrate distinct pharmacology when compared to atosiban in human myometrial smooth muscle. Atosiban displays agonist activity at micromolar concentrations leading to stimulation of prostaglandin production.

Introduction

Every year an estimated 15 million babies are born preterm (< 37 weeks of gestation) accounting for one in ten deliveries [1]. Preterm birth is the leading cause of infant mortality [2] and morbidity [3] in addition to causing a significant financial burden [4] and remains an important unmet clinical challenge. One approach to treat threatened preterm delivery has been the use of inhibitors of uterine contractility to achieve tocolysis.

Inhibitors licensed for tocolytic use include salbutamol, terbutaline, magnesium sulphate, ritodrine and atosiban [5]. Atosiban is a peptide antagonist of the vasopressin V_{1a} and OTRs [6] and is used in tocolytic treatment primarily for its actions on the OTR. Oxytocin is an important stimulator of the uterus at term [7] acting initially to activate phospholipase C via $G\alpha_{q/11}$ to stimulate production of IP_3 and the release of intracellular Ca^{2+} from the sarcoplasmic reticulum [8]. In addition to immediate Ca^{2+} release in myometrial cells oxytocin can also stimulate longer term inflammation in the myometrium and fetal membranes via nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [9], an effect mimicked by micromolar concentrations of atosiban, and blocked by the $G\alpha_i$ inhibitor, pertussis toxin [10].

The structure of atosiban is based on the peptide structure of oxytocin and it is unclear whether the second generation of small molecule antagonists, such as retosiban, which demonstrate greater selectivity for the OTR [11,12], also demonstrate agonist activity via $G\alpha_i$ at higher concentrations. Furthermore, retosiban has exhibited unexpected properties in experimental studies, intimating that its pharmacology may be different to that of atosiban. For example, retosiban inhibits stretch induced ERK signaling in myometrial explants [13]. In phase 2 trials, a single dose of retosiban significantly delayed threatened preterm labour for more than one week, suggesting a mechanism of action which outlasts the presence of the compound in the blood [14]. In the current study, we sought to determine the pharmacological mechanism of action of retosiban in comparison to atosiban using a combination of *in vitro* approaches in both human myometrium and recombinant mammalian expression systems.

Material and Methods

Ethical Approval

All procedures involving women were conducted within the guidelines of The Declaration of Helsinki and were subject to local ethical approval (REC-05/Q2802/107). Written informed consent for sample collection was obtained prior to surgery.

Subject criteria and selection

Subjects were recruited into a single group at elective caesarean section between 38-40 weeks gestation. Subjects were not in labour (NIL), as defined by an absence of observable signs of labour including regular contractions (<3min apart), membrane rupture and cervical dilatation (>2cm) with no augmentation.

Sample collection

Myometrial biopsies were collected at caesarean section by knife biopsy from the lower uterine segment incision and were obtained prior to administration of oxytocin. Samples were briefly washed in saline and flash frozen in LN_2, or placed in modified Krebs'-Henseleit (m-KHB) solution (composition (mM): NaCl; 133, KCl; 4.7, Glucose; 11.1, $MgSO_4$; 1.2, KH_2PO_4; 1.2, CaCl; 2.5, TES; 10, pH 7.4) for contraction studies.

Cell culture

Primary myometrial cell cultures were established from whole biopsies by digestion of the extracellular matrix. Primary myocytes were isolated by 2 mg/ml collagenase (Type IV, Fisher Scientific, Loughborough, UK) digestion in DMEM for 1 h at 37°C and mechanical isolation through fire-polished glass pipettes. Freshly isolated myocytes were cultured in DMEM supplemented with 10% FCS and penicillin (100 IU/ml) and streptomycin (100 μg/ml). Chinese Hamster Ovary cells recombinantly expressing the human OTR (CHO-hOTR) cells were obtained from GlaxoSmithKline and were routinely maintained in F-12 media supplemented with 10% FCS, penicillin (100 IU/ml) and streptomycin (100 μg/ml). All cells were cultured at 37°C in a 95%/5% air/CO_2-humidified environment. Cells were sub-cultured at 80% confluency by lifting with 0.05% trypsin, and not used beyond passage 3.

Organ bath studies

Muscle strips approximately 10×2×2mm were mounted vertically in 10ml organ bath chambers in m-KHB and 95% air/5% CO_2 for isometric force recordings. Force was measured with FT03C transducers (Grass Instrument Co, Quincy, MA, USA) and recorded digitally with MacLab Chart software (ADInstruments Ltd, Oxfordshire, UK). Strips were held under 20 mN tension for 90-120 min wherein rhythmical, spontaneous contractions developed. Strips that

failed to contract spontaneously were excluded. For competitive antagonism, full oxytocin-mediated concentration-response curves were generated in the presence of varying concentrations of retosiban or atosiban. Each dose of oxytocin was added for 10 min and separated by 20 min washing. Data analysis: Peak responses, peak frequency and area under the curve (integral) were calculated using MacLab Chart software from the basal and dosing periods. Changes in contractions were expressed as a percentage increase over basal and plotted using GraphPad Prism (v5.0) software.

Radioligand binding

Tissue preparation: Frozen tissue was ground to a fine powder using a pestle and mortar and homogenised in ice-cold radioligand binding buffer (composition (mM): Tris-HCl; 50, EDTA; 2.5, and $MgSO_4$; 5, pH 7.4 (with KOH)). Homogenates were cleared (1000 rpm, 10 min 4˚C), pelleted (15000 rpm, 15 min, 4˚C) and resuspended in radioligand binding buffer where protein was adjusted to 1 mg/ml. Radioligand binding assay: Assays were performed in 500 µl volumes of radioligand binding buffer containing ~100 µg membranes and [^{3}H]-Oxytocin (SA: 50 µCi/mmol) (Perkin Elmer. Massachusetts, U.S.A.) at concentrations ranging from 0.001-50 nM. Non-specific binding was determined by the inclusion of 100 nM unlabeled oxytocin. Assays were equilibrated at 30˚C for 2h before harvest through 0.5% polyethylenimine pre-soaked Whatman GF/B filters. Recovered radiation was determined by standard liquid scintillation counting. Data analysis: Specific binding was determined as total binding less non-specific binding. Data were fitted using GraphPad Prism (v5.0) and the B_{max} and K_D values obtained.

Measurement of cAMP

cAMP was determined using the 2-step HTRF cAMP kit (Cisbio Bioassay, Codolet, France). Cell culture and agonist stimulation: Cells were seeded in 96-well plates at a density 20000 cells/well and, where applicable, treated with 100 ng/ml pertussis toxin (PTX) for 18h. Cells were washed in m-KHB containing 300 µM 3-isobutyl-1-methylxanthine (IBMX) and equilibrated in buffer at 37˚C for 15 min. For $G\alpha_s$ experiments, cells were stimulated directly with compounds as required, and for assessment of $G\alpha_i$ signaling, cells were stimulated for time period indicated prior to challenge with forskolin (FSK) (10 µM, 10 min). Assays were terminated by addition of 25 µl kit-supplied lysis buffer. Detection of cAMP: 10 µl from each well was transferred in duplicate to white, low volume 384-well plates (Corning®) and detection of cAMP proceeded exactly as per manufacturer's instructions (CisBio) with fluorescence determined on a PHERstar FS plate reader (BMG Labtech). Data analysis: cAMP

levels in each well were interpolated from a 4-parameter fit of known standards (Microsoft Excel) and plotted graphically using GraphPad Prism (v5.0).

Measurement of phosphorylated ERK (pERK) in cells

Levels of phosphorylated ERK were determined using the HTRF Cellul'ERK assay kit (Cisbio Bioassay, Codolet, France) as per manufacturer's instructions. Cell stimulation and lysis: Myometrial cells were seeded in 96-well plates at a density 5000 cells/well and cultured overnight before serum-starvation for 24h. Where appropriate, cells were pre-treated with 100 ng/ml PTX for 18h. Cells were washed in m-KHB and equilibrated in buffer at 37°C for 30min. Cells were challenged with compounds for time points indicated before aspiration and addition of 50 µl supplied-lysis buffer (containing phosphatase inhibitors) for 30 min (RT). Detection of pERK: 16 µl of each well was transferred in duplicate to white, low volume 384-well plates (Corning®) for detection of pERK as per the Cellul'ERK assay manufacturer's instructions with fluorescence determined on a PHERstar FS plate reader. Data analysis: Increases in pERK were calculated as % increase over basal (vehicle, unstimulated) cells.

Measurement of PGE_2, $PGF_{2\alpha}$ and protein kinase A (PKA) in myometrial strips

Muscle strips approximately 5×2×2 mm were mounted horizontally in a DMT Flatbed muscle strip myograph system (DMT, Hinnerup, Denmark). Strips were held under 20 mN tension in m-KHB at 37°C wherein spontaneous contractions occurred. Drugs were added directly into the organ bath as per protocol requirements. Tissue was snap frozen by rapid removal from organ baths and immediate submersion into LN_2. Concurrently, aliquots of the supernatant were collected for assessment of prostaglandin production. Where necessary, tissue biopsies were treated with 100 ng/ml PTX overnight at 4°C to inhibit $G\alpha_i$ signaling, or 1h pre-treatment with 10 µM U0126 to inhibit ERK signaling or 10 µM rofecoxib to inhibit COX-2. Preparation of cell lysates: Frozen tissue was diced into small pieces before homogenisation in 1 ml RIPA buffer containing protease (Pierce™ tablets) and phosphatase (phosphatase inhibitor cocktail 2 (diluted 1:100), (Sigma, Poole, UK)) inhibitors. Homogenisation was achieved using a NEXT Advanced BBY24M Bullet Blender Storm (Next Advanced, Averill Park, NY, USA) for 10min at 4°C, and the NAVY ball bearing mix (refer to manufacturer's instructions). Lysates were subject to clearance (500 rpm, 2 min, 4°C) before transfer to duplicate tubes for assay and protein quantification via a modified Lowry assay (DC™ Protein Assay, Bio-Rad, CA, U.S.A.). Quantitation of PGE_2 and $PGF_{2\alpha}$ in tissue supernatant was via competitive ELISA exactly as per manufacturers' instructions. The PGE_2 assay (KGE0048) was obtained from R&D Systems (Minneapolis, MN, USA), and the $PGF_{2\alpha}$ assay (516011) from Cayman

Chemicals (Ann Arbor, MI, USA). Tissue homogenates were assessed for PKA using a PKA activity assay from Arbor Assays (K027-H1) and COX-2 by sandwich ELISA using (DYC4198-2) from R&D Systems (Minneapolis, MN, USA). Data analysis: In all cases, samples were extrapolated from known standards using a 4-parameter fit curve in GraphPad prism. PGE_2 and $PGF_{2\alpha}$ production, PKA activity and COX-2 levels were related to protein content.

[^{35}S]-guanosine 5'-O-[gamma-thio]triphosphate ([^{35}S]GTPγS) binding

[^{35}S]-GTPγS is a non-hydrolyzable analog of GTP that stays bound to activated G-proteins. Specific G-protein activation can therefore be detected by virtue of the radiolabel, and co-immunoprecipitation with specific Gα antibodies. The [^{35}S]-GTPγS assay was performed according to a modification of methodologies described previously[15-18] Membrane preparation: Primary myometrial cultures and CHO-hOTR cells were grown to confluence in 175cm^2 flasks, lifted and pelleted via standard cell culture techniques. Pelleted cells were homogenized in the presence of hypotonic lysis buffer (10 mM EDTA, 10 mM HEPES, pH 7.4) using a Coleman handheld homogenizer. The homogenate was pre-cleared by centrifugation (500 g, 5 min, 4°C) and the membranes collected via centrifugation (36,000g, 30 min, 4°C). Membranes were resuspended in freezing buffer (10 mM HEPES, 0.1 mM EDTA, pH 7.4) at 6 mg/ml protein and rapidly frozen in liquid nitrogen. Membranes were stored at -80°C until used. Receptor activation: Frozen membrane aliquots were diluted to 1.5 mg/ml in assay buffer (composition: (mM): HEPES; 10, NaCl; 100, $MgCl_2$; 10, pH 7.4) and 75 µg membrane were added to 50 µl of assay buffer containing 1 nM [^{35}S]-GTPγS (1250 Ci mmol^{-1}) and 1 µM GDP, with or without ligands as required, and incubated at 30°C for 2 min. Non-specific binding was determined by the inclusion of 10 µM unlabeled GTPγS. Incubations were terminated by 900 µl ice-cold assay buffer and transfer to ice. Cell membranes were recovered from the reaction mixture by centrifugation (20,000g, 6 min, 4°C) and the supernatant removed by aspiration. Membrane pellets were solubilized by the addition of 50 µl ice-cold solubilization buffer (composition (mM): Tris/HCl; 100, NaCl; 200, EDTA; 1 and 1.25% (v:v) Igepal CA 630, pH 7.4) containing 0.2% (w:v) SDS and vortex-mixing. Once the protein was completely solubilized, an equal volume of solubilization buffer without SDS was added. Immuno-detection of G-Proteins: The solubilized protein was pre-cleared with normal rabbit serum (1:100 dilution) and 30 µl of Protein-G sepharose beads (protein-G sepharose bead suspension 30% w:v in TE buffer (10 mM Tris/HCl, 10 mM EDTA, pH 8.0)) for 60 min at 4°C. The protein-G beads and any insoluble material were collected by centrifugation

(20,000g, 6 min, 4°C) then 100 µl of the supernatant was transferred to a fresh tube containing G-protein antiserum (1:100 dilution). Samples were vortex-mixed and rotated overnight at 4°C. To each sample tube was added 70 µl of Protein G-sepharose bead suspension and the samples vortex-mixed and rotated for 90 min at 4°C. Protein G-sepharose beads were pelleted at 20,000 g and supernatant removed. Beads were washed and pelleted three times with 500 µl solubilization buffer (less SDS) before re-suspension in scintillation cocktail where [^{35}S]-GTPγS was determined by standard liquid scintillation counting methods. Data analysis: CPM values were expressed as a % increase over basal (unstimulated).

IP-One assay

The HTFR IP-One assay was performed exactly as per manufacturer's instruction (CisBio). Briefly, Cells were seeded at 50,000 cells well^{-1} in white, low volume, 384-well plates (Corning®). On day of assay, cells were washed with stimulation buffer (provided with kits) and left with 7 µl buffer per well. Duplicate ligand plates were made containing double the desired concentrations of antagonist, agonist or vehicle as required and cells stimulated by direct transfer of 7 µl to corresponding wells on the assay plate. Cells were stimulated for 1 h, at 37°C, before reactions were terminated and fluorescence determined on a PHERstar FS plate reader (BMG Labtech). Data analysis: IP-1 levels in each well were interpolated from a 4-parameter fit of known standards (Microsoft Excel) and plotted graphically using GraphPad Prism (v5.0), from where EC_{50} values were obtained. To determine antagonist potency, pA_2 values were determined from the intercept when y=0 using the Schild equation (Log (EC_{50} ratio -1) vs. Log [antagonist]).

Measurement of IP_3 in myometrial strips

Muscle strips were mounted horizontally in a flatbed muscle strip myograph as described above with drugs added directly into the organ bath. Tissue was snap frozen at critical points by rapid removal from baths and immediate submersion into LN_2. Critical points included the peak of a spontaneous contraction, midway between spontaneous contractions, peak of oxytocin-mediated contraction and during inhibition of contraction in the presence of retosiban and atosiban. Frozen tissue was mechanically ground into a fine powder by pestle and mortar and homogenized in 1M trichloroacetic acid (TCA) for 10 min at 4°C in a Bullet Blender Storm (Next-Advanced, New York, USA). 400 µM TCA was transferred to tubes and 50 µl 10 mM EDTA added. TCA was extracted by addition of 1 ml 1:1 (v:v) dilution of tri-n-octylamine:1,1,2-trichlorotrifluoroethane and vigorous mixing. After 10 min, tubes were centrifuged (1000 rpm 2 min, RT) and 400 µl of top aqueous phase transferred to duplicate tubes where 50 µl 50mM $NaHCO_3$ was added. Determination of IP_3. IP_3 levels were determined

using the [^{3}H]-IP_3 Radioreceptor assay kit (PerkinElmer Life Inc, Boston, MA) as per the manufacturer's instructions. All dilutions were considered and IP_3 was calculated in pmol/mg protein.

Protein determination:

Unless otherwise stated, protein content was assessed by the Bradford method [19].

Statistical analysis

Contractility studies:

Experiments were repeated on biopsies obtained from different women where n represents the number of biological replicates. For figures 1 and 2 data were analysed by one-way ANOVA followed by Dunnett *post hoc* test comparing test compound to vehicle, and $P < 0.05$ considered significant. For figure 3 data were analysed by mixed model ANOVA with antagonist as the between measures variable followed by Dunnett *post hoc* test comparing test antagonist concentration to vehicle, and $P < 0.05$ considered significant. *P* values were graphically represented as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. For figure 4 data were analysed by paired T-Test comparing observations before and after retosiban treatment, and $P < 0.05$ considered significant.

IP1 assay:

Experiments were repeated on CHO-hOTR cells where n represents the number of technical replicates. For figure 5 data were analysed by mixed model ANOVA with antagonist as the between measures variable followed by Dunnett *post hoc* test comparing test antagonist concentration to vehicle, and $P < 0.05$ considered significant. *P* values were graphically represented as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. IP-1 levels were interpolated from a 4-parameter fit of known standards and plotted graphically for calculations of EC_{50} values using GraphPad Prism (v5.0). For antagonist potency, pA_2 values were determined from the intercept when y=0 using the Schild equation (Log (EC_{50} ratio -1) vs. Log [antagonist]).

IP_3 assay:

Experiments were repeated on myometrial membrane preparations from different women where n represents the number of biological replicates. For figure 6 data were analysed by one-way ANOVA followed by Dunnett *post hoc* test comparing test compound to vehicle, and $P < 0.05$ considered significant. Refractory period and peak contraction were analysed separately. *P* values were graphically represented as * $P < 0.05$, ** $P < 0.01$.

cAMP assay:

Experiments were repeated on CHO-hOTR cells where n represents the number of technical replicates. For figure 7 data were analysed by two-way ANOVA followed by Dunnett *post hoc*

test comparing test compound time series to vehicle, and $P < 0.05$ considered significant. *P* values were graphically represented as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

ERK assay:

Experiments were repeated on primary human myometrial cultures derived from different patient biopsies where n represents the number of biological replicates. For figure 8 data were analysed by two-way ANOVA followed by Dunnett *post hoc* test comparing test compound time series to vehicle, and $P < 0.05$ considered significant. *P* values were graphically represented as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

COX-2, PKA, PGE_2 and $PGF_{2\alpha}$ assay:

Experiments were repeated on biopsies obtained from different women where n represents the number of biological replicates. For figure 9 data were analysed by two-way ANOVA followed by Dunnett *post hoc* test comparing test compound time series to vehicle, and $P < 0.05$ considered significant. *P* values were graphically represented as * $P < 0.05$, ** $P < 0.01$. For figure 10 data were analysed by one-way ANOVA with atosiban and oxytocin analysed separately followed by Dunnett *post hoc* test comparing inhibitor compound to oxytocin or atosiban alone, and $P < 0.05$ considered significant. *P* values were graphically represented as * $P < 0.05$, ** $P < 0.01$.

G-protein coupling assay:

Experiments were repeated on membrane preparations from primary human myometrial cultures derived from different patient biopsies where n represents the number of biological replicates. For figure 11 data were analysed by one-way ANOVA followed by Dunnett *post hoc* test comparing test compound to vehicle, and $P < 0.05$ considered significant. *P* values were graphically represented as * $P < 0.05$, ** $P < 0.01$.

Results

Prior to detailed studies of the OTR signaling in myometrial strips, we first characterized the basic physiological responses to oxytocin. The addition of increasing concentrations of oxytocin to human myometrial smooth-muscle strips evoked a concentration-dependent increase in the magnitude, integral and frequency of spontaneous contractions (**Fig. 1a and b**), whereas no changes were observed by addition of vehicle control (**Fig. 1b**). Concentration-response analysis (**Fig. 1b**) revealed EC_{50} values of: peak -8.224 ± 0.46 (5.9 nM), integral -8.053 ± 0.13 (8.8 nM), and frequency -8.229 ± 0.16 (5.0 nM). In radioligand binding studies, membranes were prepared from whole muscle biopsies and saturated with [^{3}H]-oxytocin to permit the quantification of OTR expression. The binding of [^{3}H]-oxytocin to membranes prepared from biopsies was saturable. Specific binding, as determined by inclusion of 100nM oxytocin represented approximately 40% of the total binding at a K_D concentration of [^{3}H]-oxytocin. Analysis of saturation binding curves indicated a B_{max} value of 8337 ± 931 fmol/mg protein, and a K_D value of 24.15 ± 6.5nM for oxytocin (**Fig. 1c**).

The saturation studies confirmed previous studies that the OTR reaches high concentrations in the myometrium at term [20,21]. Under such conditions it is feasible that unliganded receptor contributes to downstream signaling. Taken together with previous studies suggesting that retosiban may be an inverse agonist (defined as a ligand that can reduce constitutive receptor activity)[13], we sought to investigate whether retosiban could reduce spontaneous contractions in the absence of oxytocin. The addition of retosiban (**Fig. 2a**) or atosiban (**Fig. 2b**) did not significantly reduce the mean of magnitude, activity integral, or frequency of contractions in spontaneously contracting strips. In competition assays using oxytocin as agonist, however, the presence of increasing concentrations of retosiban evoked a right-ward shift in oxytocin concentration-response curve for peak (**Fig. 3a**), integral (**Fig. 3b**) and frequency (**Fig. 3c**). Similar observations were made with atosiban (**Fig. 3d-f**). EC_{50} values observed are displayed in table 1.

Data from a phase 2 clinical trial suggest that retosiban may have a long acting mechanism of action [14]. To test whether retosiban remained active at the receptor for prolonged periods we challenged tissues with oxytocin immediately after washout of a preincubation with retosiban. The actions of retosiban were rapidly reversible. Removal of retosiban by washing and immediate addition of 100nM oxytocin resulted in contractile responses that were instant (**Fig. 4a**), and of similar magnitude to those preceding retosiban addition (**Fig. 4b**).

To investigate potential for ligand bias (defined as a ligand specific selectivity for a downstream signaling pathway) from the OTR antagonists, we systematically investigated

their mechanism of downstream signaling and potency. To confirm that any ligand bias was not simply a myometrial cell phenomenon we first tested the effect of the OTR antagonists on a Chinese Hamster Ovary cell line stably expressing the human oxytocin receptor. Oxytocin generated concentration-dependent increases in IP-1 accumulation (**Fig. 5a-c**), a stable metabolite of IP_3. Maximal responses were achieved at 100μM oxytocin with an EC_{50} value of -6.48±0.17 (332nM). The presence of increasing concentrations of retosiban (**Fig. 5a**), atosiban (**Fig. 5b**), and epelsiban an additional potent small molecule inhibitor of the OTR [22,23] provoked a right-ward curve shift (**Fig. 5c**), whereby increasing concentrations of oxytocin were required to illicit the same responses. pA_2 values (the dose of antagonist needed to shift the agonist dose-response curve 2-fold to the right) were obtained using the Schild equation (**Fig. 5d**). Of the 3 antagonists, epelsiban was most potent with a pA_2 value of -8.537, followed by retosiban -8.045 and finally atosiban -6.159. The data would indicate an increase in potency of inhibition of IP_3 generation as approximately 77-fold for retosiban and 240-fold for epelsiban when compared to atosiban. Further analysis of Schild plots revealed slopes of 1.017, 1.069 and 1.042 for epelsiban, retosiban and atosiban, respectively. pA_2 and slope values are detailed in Table 2.

To investigate IP_3 production in intact tissue, uterine strips were treated with various compounds, and snap frozen either mid-way between or at the peak of contractions and IP_3 content determined. Initial comparisons of contraction versus inter-contraction interval demonstrated IP_3 levels were essentially the same irrespective of contractions (**Fig. 6a)**. By contrast 100nM oxytocin markedly increased IP_3 levels (**Fig. 6a**), whilst IP_3 was significantly reduced in the presence of 1μM retosiban (**Fig. 6a**). To investigate and confirm this effect further we increased the number of observations during peak contraction whereby retosiban again decreased IP_3 when compared to vehicle control whereas IP_3 levels in 10μM atosiban-treated strips decreased but did not reach significance (**Fig.6b**).

To further investigate downstream signaling and potential ligand bias at the OTR we challenged CHO-*h*OTR cells with either oxytocin, atosiban, retosiban or epelsiban and measured cAMP accumulation. None of the ligands elevated levels of cAMP (**Fig. 7a**), whereas addition of either forskolin or calcitonin, a selective $G\alpha_s$-coupled receptor agonist, caused a robust and saturable elevation of cAMP accumulation. These data suggest that the OTR ligands do not signal through $G\alpha_s$ G-proteins in these cells.

Both oxytocin and atosiban reduced calcitonin (**Fig. 7b**) and forskolin mediated cAMP production (**Fig. 7c**), providing evidence of $G\alpha_i$-coupling. The potent and selective 5-HT_{1B} ($G\alpha_i$-coupled) agonist CP93129 [24] evoked similar changes. Importantly, neither retosiban

nor epelsiban had any effect on cAMP. The oxytocin and atosiban mediated inhibition of cAMP were PTX sensitive demonstrating $G\alpha_i$ involvement (**Fig. 7d**).

Stimulation of $G\alpha_i$ signaling in myometrial cells has the potential to activate ERK [25]. We therefore tested whether challenge of cultured myometrial cells taken at term cesarean section with either oxytocin or atosiban evoked an increase in ERK phosphorylation. Application of either oxytocin or atosiban increased ERK activity in a concentration-dependent manner (**Fig. 8a**) and generated EC_{50} values of -6.93 ± 0.34 (118nM) and -5.10 ± 0.32 (8.03μM) for oxytocin and atosiban respectively that peaked at ~5 min and subsequently declined (**Fig. 8b**). Cells did not respond to either controls, retosiban or epelsiban at equivalent receptor occupancy. Oxytocin and atosiban-mediated responses were inhibited by preincubation with pertussis toxin (PTX) (**Fig. 8c**).

Increases in ERK activity in myometrial smooth muscle are associated with increased production of prostaglandins. We observed an increase in COX-2 (**Fig. 9a**) expression, and PGE_2 (**Fig. 9c**) and $PGF_{2\alpha}$ (**Fig. 9d**) secretion on challenge of spontaneously contracting myometrial strips with oxytocin and atosiban, but not retosiban or epelsiban. COX-2 expression increased rapidly, but PGE_2 and $PGF_{2\alpha}$ secretion was absent until 2hr where a 3 to 4-fold increase was observed. The magnitude of responses was similar for both oxytocin and atosiban. No increases in protein kinase A (PKA) activity were detected (except with the positive control forskolin), and no reduction in basal PKA levels were observed (**Fig. 9b**). PGE_2 (**Fig. 10a**) and $PGF_{2\alpha}$ (**Fig. 10b**) secretion was inhibited by PTX ($G\alpha_i$ inhibition), U0126 (ERK inhibition) and rofecoxib (COX-2 inhibition). COX-2 expression was inhibited by PTX and U0126 (**Fig. 10c**).

The downstream signaling analysis of the ligands suggested that contrary to atosiban being a neutral antagonist, it is in fact, an agonist at $G\alpha_i$ at micromolar concentrations. To investigate coupling of the OTR to specific Gα-subunits we utilised the [^{35}S]-GTPγS immunoprecipitation assay in membrane preparations from cultured myometrial cells taken from patients at term. Oxytocin increased [^{35}S]-GTPγS binding to $G\alpha_{q/11}$ (**Fig. 11a**) G-proteins and both oxytocin and atosiban increased binding of $G\alpha_i$ subunits (**Fig. 11b**). Consistent with cAMP data no binding was observed following immunoprecipitation with $G\alpha_s$ G-proteins (**Fig. 11c**). Combined with signaling analysis these data confirm that at micromolar concentrations atosiban couples OTR to $G\alpha_i$ to elicit activation of ERK in human myometrial cells. Importantly, neither retosiban nor epelsiban elicited any increases in [^{35}S]-GTPγS binding, signifying no agonist activity with these compounds.

Discussion

This study demonstrates that there are significant differences in the pharmacology of two small molecule OTR antagonists, retosiban and epelsiban, when compared to atosiban, a peptide antagonist licensed for use as a tocolytic for the treatment of preterm labour. The observations of this study, summarized in Figure 12 explain some, but not all, of the observed physiological effects of retosiban in the published literature. In a phase 2 trial, administration of intravenous retosiban as a single dose was sufficient to inhibit spontaneous preterm birth by more than one week [14]. Our data suggest that it is highly unlikely that the long-lasting effect of the single dose was a consequence of continued action at the OTR, such as has been previously observed for the β_2-adrenergic receptor agonist salmeterol [26], since antagonism of oxytocin stimulated contractions in uterine strips was rapidly reversible.

All three antagonists tested in this study effectively inhibit OTR coupling to $G\alpha_{q/11}$ and the generation of IP_3, with epelsiban being the most potent, followed by retosiban and atosiban. Since stimulation of Ca^{2+} release from the sarcoplasmic reticulum by IP_3 is the first component of the mechanism of oxytocin's rapid action on the uterus [27] administration of all compounds should result in inhibition of the initial contractile effect. In addition to an effect on oxytocin mediated IP_3 signaling, the addition of retosiban reduced IP_3 in intact strips under basal conditions. Such a reduction is consistent with inverse agonism although it is difficult to rule out the presence of endogenous OT bound to recycled OTR in myometrial samples or endosomal signaling during altered trafficking.

Previous work on atosiban has demonstrated that at micromolar concentrations the molecule acts as an agonist stimulating $G\alpha_i$ and causes inflammation in amnion cells [9,10]. In this study we demonstrated that in the myometrium atosiban at micromolar concentrations stimulates coupling of the OTR to $G\alpha_i$ and subsequent phosphorylation of ERK 1/2, COX2 upregulation, and PGE_2 and $PGF_{2\alpha}$ secretion. No such stimulation was observed with the addition of retosiban. In CHO-OTR cells addition of micromolar atosiban or nanomolar oxytocin inhibited calcitonin and forskolin stimulated cAMP production, an effect that was prevented by addition of pertussis toxin, suggesting that the observed ligand bias is not cell specific.

The functional consequences of the ligand bias of atosiban *in vivo* are hard to interpret without accurate tissue concentrations. Tissue concentrations that reach, or exceed, micromolar levels are likely to elicit inflammation and release of prostaglandins some hours after treatment. Thus, the tocolytic effect mediated by inhibition of $G\alpha_{q/11}$ may initially be effective in inhibiting

443 contractions but after some hours would paradoxically make contractions more likely via
444 agonism of the OTR and stimulation of $G\alpha_i$.
445 We conclude that the small molecule oxytocin receptor antagonists retosiban and epelsiban
446 inhibit downstream signaling in myometrial cells and CHO-hOTR cells in a manner consistent
447 with that of a neutral antagonist. By contrast, the peptide based, mixed oxytocin/vasopressin
448 V_{1a} receptor antagonist Atosiban, inhibits oxytocin receptor signaling through the $G\alpha_{q/11}$ g
449 protein but acts as a partial agonist for signaling through the $G\alpha_i$ pathway.
450

Tables.

Table 1.

Retosiban effects on peak contractions (Fig 3a)								
Parameter	**Vehicle**	**1nM retosiban**	**3nM retosiban**	**10nM retosiban**	**30nM retosiban**	**100nM retosiban**	**300nM retosiban**	**1000nM retosiban**
LogEC$_{50}$ ± sem	-8.67 ± 0.34	-9.34 ± 0.56	-9.484 ± 0.57	-9.345 ± 0.44	-9.302 ± 0.35	-8.251 ± 0.0.47	-8.771 ± 0.75	-8.546 ± 0.77
p value (mixed model ANOVA)	ns	ns	ns	ns	0.014	<0.0001	<0.0001	<0.0001
E$_{max}$ ± sem	132 ± 3.49	123.9 ± 5.26	129.5 ± 5.78	129.2 ± 5.60	125.6 ± 6.66	118.3 ± 11.31	111.7 ± 9.07	107.7 ± 10.91
CI (LogEC$_{50}$)	-9.358 to -8.000	-10.42 to -8.281	-10.62 to -8.346	-10.18 to -8.573	-9.991 to -8.613	-9.197 to -7.305	-10.26 to -7.281	-10.07 to -7.024

Retosiban effects on integral (Fig 3b)								
Parameter	**Vehicle**	**1nM retosiban**	**3nM retosiban**	**10nM retosiban**	**30nM retosiban**	**100nM retosiban**	**300nM retosiban**	**1000nM retosiban**
LogEC$_{50}$ ± sem	-8.354 ± 0.28	-8.641 ± 0.28	-8.489 ± 0.37	-8.614 ± 0.40	-8.269 ± 0.22	-8.048 ± 0.34	-8.159 ± 0.27	-7.575 ± 0.31
p value (mixed model ANOVA)	ns	ns	ns	ns	<0.0001	<0.0001	<0.0001	<0.0001
E$_{max}$ ± sem	414 ± 34.37	368.6 ± 30.69	366.7 ± 41.23	340 ± 38.85	331 ± 30.99	325.6 ± 53.21	302.3 ± 35.91	298 ± 54.06
CI (LogEC$_{50}$)	-8.908 to -7.801	-9.191 to -8.092	-9.229 to -7.750	-9.411 to -7.817	-8.714 to -7.823	-8.734 to -7.363	-8.706 to -7.612	-8.193 to -6.956

Retosiban effects on contraction frequency (Fig 3c)								
Parameter	**Vehicle**	**1nM retosiban**	**3nM retosiban**	**10nM retosiban**	**30nM retosiban**	**100nM retosiban**	**300nM retosiban**	**1000nM retosiban**
LogEC$_{50}$ ± sem	-7.742 ± 0.53	-7.992 ± 0.36	-7.981 ± 0.30	-7.589 ± 0.50	-7.599 ± 0.50	-7.576 ± 0.49	-6.988 ± 1.16	-7.162 ± 1.51
p value (mixed model ANOVA)	ns	ns	ns	ns	ns	ns	0.0006	0.0036
E$_{max}$ ± sem	228.3 ± 37.55	233.8 ± 26.61	235.6 ± 19.62	206 ± 34.48	201 ± 32.44	184.2 ± 39.62	194 ± 137.1	156 ± 743.67
CI (LogEC$_{50}$)	-8.802 to -6.683	-8.713 to -7.271	-8.580 to -7.382	-8.580 to -6.598	-8.597 to -6.601	-8.558 to -6.593	-9.292 to -4.684	-10.16 to -4.162

Atosiban effects on peak contractions (Fig 3d)								
Parameter	**Vehicle**	**0.01µM Atosiban**	**0.03µM Atosiban**	**0.1µM Atosiban**	**0.3µM Atosiban**	**1µM Atosiban**	**3µM Atosiban**	**10µM Atosiban**
LogEC$_{50}$ ± sem	-8.449 ± 0.20	-9.152 ± 0.40	-8.919 ± 0.31	-8.826 ± 0.24	-8.826 ± 0.34	-8.221 ± 0.40	-8.443 ± 0.49	-7.784 ± 0.58
p value (mixed model ANOVA)	ns	ns	ns	ns	ns	0.0056	<0.0001	<0.0001
E$_{max}$ ± sem	137.6 ± 3.3	135.8 ± 4.53	143.2 ± 4.54	139.6 ± 3.70	128 ± 5.24	126.4 ± 6.63	117.7 ± 7.43	118.3 ± 12.36
CI (LogEC$_{50}$)	-8.836 to -8.063	-9.949 to -8.355	-9.538 to -8.299	-9.298 to -8.354	-9.532 to -8.115	-9.025 to -7.417	-9.412 to -7.474	-8.947 to -6.622

Atosiban effects on integral (Fig 3e)								
Parameter	**Vehicle**	**0.01µM Atosiban**	**0.03µM Atosiban**	**0.1µM Atosiban**	**0.3µM Atosiban**	**1µM Atosiban**	**3µM Atosiban**	**10µM Atosiban**
LogEC$_{50}$ ± sem	-8.327 ± 0.15	-8.835 ± 0.37	-9.121 ± 0.20	-8.932 ± 0.24	-8.47 ± 0.24	-8.239 ± 0.29	-8.071 ± 0.28	-7.97 ± 0.21
p value (mixed model ANOVA)	ns	ns	ns	ns	ns	0.0057	<0.0001	<0.0001
E$_{max}$ ± sem	410.6 ± 23.37	326.7 ± 27.41	346.3 ± 18.16	311.1 ± 19.23	296.7 ± 23.17	268 ± 26.54	222.3 ± 21.42	251.3 ± 22.02
CI (LogEC$_{50}$)	-8.624 to -8.029	-9.501 to -8.180	-9.519 to -8.723	-9.402 to -8.463	-8.952 to -7.987	-8.810 to -7.668	-8.627 to -7.516	-8.394 to -7.546

Atosiban effects on contraction frequency (Fig 3f)								
Parameter	**Vehicle**	**0.01µM Atosiban**	**0.03µM Atosiban**	**0.1µM Atosiban**	**0.3µM Atosiban**	**1µM Atosiban**	**3µM Atosiban**	**10µM Atosiban**
LogEC$_{50}$ ± sem	-8.033 ± 0.31	-8.154 ± 0.45	-7.096 ± 0.47	-7.775 ± 0.25	-7.311 ± 0.65	-7.732 ± 0.67	-7.502 ± 0.36	-7.058 ± 1.21
p value (mixed model ANOVA)	ns	ns	ns	0.0223	ns	0.0342	<0.0001	<0.0001
E$_{max}$ ± sem	224.9 ± 18.36	225.3 ± 26.42	315.5 ± 106.4	214.6 ± 19.37	227.6 ± 70.24	179.4 ± 25.87	166.8 ± 17.99	178 ± 98.89
CI (LogEC$_{50}$)	-8.653 to -7.414	-9.055 to -7.254	-8.037 to -6.154	-8.276 to -7.274	-8.616 to -6.007	-8.874 to -6.574	-8.214 to -6.790	-9.467 to -4.649

Table 2.

Antagonist	**Epelsiban**	**Retosiban**	**Atosiban**
pA$_2$ (Log$_{10}$ M)	-8.537	-8.045	-6.159
pA$_2$ (nM)	2.9	9.02	698.23
Slope ± sem	1.017 ± 0.105	1.069 ± 0.105	1.042 ± 0.096

Figure Legends:

Figure 1.

(**a**) Representative trace showing phasic myometrial contractions in response to increasing concentrations of oxytocin (0.01nM-100nM). (**b**) Oxytocin (red square) concentration-response curves. Data were analysed to assess the peak responses (peak-minimum), the area under the curve (integral) and the peak frequency (Hz) during the 10 min oxytocin stimulation. Data were expressed as a percentage increase over basal, unstimulated contractions in the presence of vehicle (black circle). Data revealed EC_{50} values of peak: -8.224 ± 0.46 (5.9nm), integral: -8.053 ± 0.13 (8.8nm) and peak frequency: -8.229 ± 0.16 (5.0nm). Data are mean ± s.e.m., n=3 (One-Way ANOVA and Dunnett's Multiple Comparison Test comparing oxytocin treatment to vehicle control, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) (**c**) Saturation binding in membranes prepared from myometrial biopsies incubated with varying concentrations of [^{3}H]-Oxytocin (0.01pM-100nM) either in the presence (non-specific binding) or absence (total binding) of 100nM unlabeled oxytocin. B_{max} and K_D were 8337 ± 931 fmol/mg protein, and 24.15 ± 6.5nM, respectively. Data are mean ± s.e.m., n=6

Figure 2.

Spontaneously contracting myometrial biopsies were challenged with increasing concentrations of either retosiban (red square) (**a**) or atosiban (blue triangle) (**b**). Data were expressed as a percentage increase of basal, unstimulated contractions. Neither retosiban nor atosiban had significant effect on spontaneous contractions when compared to vehicle control (One-Way ANOVA and Dunnett's Multiple Comparison Test). Data are mean ± s.e.m., n=10

Figure 3.

Antagonist/agonist competition assays. Full oxytocin concentration-response curves were performed in the continued presence of either vehicle, or various concentrations of retosiban (0.001μM - 1μM) (**a-c**) or atosiban (0.01μM - 10μM) (**d-f**). Oxytocin doses were added for 10 minutes and separated by a 20 minutes wash period during which the desired concentration of retosiban was continually present. Data were analysed in 10 minute blocks to assess the peak responses (peak-minimum) (***a and d***), the area under the curve (integral) (***b and e***) and the peak frequency (Hz) (***c and f***). Data were expressed as a percentage increase of basal, unstimulated contractions. Data are mean ± s.e.m., n=10 (data were analysed by mixed model ANOVA with antagonist as the between measures variable followed by Dunnett *post hoc* test comparing test

antagonist concentration to vehicle, and $P < 0.05$ considered significant * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). EC_{50} and E_{max} values obtained are shown in table 1.

Figure 4.

Reversibility of retosiban antagonism (**a**) Representative traces showing the immediately reversible effects of 100nm oxytocin and 1μM retosiban on myometrial muscle strips. (**b**) Peak responses (peak-minimum), the area under the curve (integral) and the peak frequency (Hz) were expressed as a percentage increase of basal, unstimulated contractions. Colours and symbols denote paired observations within a biological replicate. Data are mean ± s.e.m., n=3 (data were analysed by paired T-Test comparing observations before and after retosiban treatment, and $P < 0.05$ considered significant).

Figure 5.

Oxytocin-mediated IP-1 accumulation in CHO-hOTR cells. Cells were stimulated for 1hr with oxytocin in the presence of increasing concentrations of retosiban (**a**) atosiban (**b**) or epelsiban (**c**). Data are mean ± s.e.m, n=3 (data were analysed by mixed model ANOVA with antagonist as the between measures variable followed by Dunnett *post hoc* test comparing test antagonist concentration to vehicle, and $P < 0.05$ considered significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). For Schild plot analysis of antagonist activity EC_{50} values were converted using Schild equation (Log (EC_{50} ratio-1) vs. Log [antagonist]) and pA_2 values obtained where y=0 (**d**). pA_2 and slope analysis values observed are indicated in table 2.

Figure 6.

IP_3 levels in frozen muscle strips. (**a**) Tissues were frozen in LN_2 between contractions (refractory period) or at the peak of contraction in the presence of vehicle, 1μM retosiban, 10μM atosiban or 100nM oxytocin. IP_3 levels in oxytocin-challenged muscle strips were significantly greater when compared to vehicle. The addition of 1μM retosiban significantly reduced IP_3 when compared to vehicle. mean ± s.e.m., n=5 (data were analysed by one-way ANOVA followed by Dunnett *post hoc* test comparing test compound to vehicle, and $P < 0.05$ considered significant. Refractory period and peak contraction were analysed separately, * $P < 0.05$, ** $P < 0.01$) (**b**) To confirm the observation that retosiban reduced IP_3 levels in spontaneously contracting strips we repeated the experimental protocol in (**a**) with more numbers, measuring IP_3 during the refractory period. Again, retosiban significantly reduced IP_3. mean ± s.e.m., n=10.

Figure 7.

(**a**) CHO-*h*OTR cells were challenged with compounds for time points indicated and lysed. Data are mean ± s.e.m., n=3. (**b**) CHO-hOTR cells were challenged simultaneously with compound and 10nM calcitonin for time points indicated before lysis. Inhibition of cAMP by oxytocin, atosiban and CP93129 (a potent and selective 5-HT1B receptor ($G_{\alpha i}$-coupled) agonist) was significant when compared to calcitonin + vehicle. Data are mean ± s.e.m., n=3. (**c**) CHO-hOTR cells were stimulated with compounds for time points indicated before FSK challenge (10µM, 10min). Atosiban, oxytocin and CP93129 reduced FSK elevated cAMP when compared to vehicle. Data are mean ± s.e.m., n=3 (data were analysed by two-way ANOVA followed by Dunnett *post hoc* test comparing test compound time series to vehicle, and $P < 0.05$ considered significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). (**d**) As (**c**) but cells were treated with either 100ng/ml PTX or vehicle for 18hrs prior to stimulus. PTX pre-treatment reversed the inhibition of cAMP production by oxytocin atosiban and CP93129.

Figure 8.

(**a**) Cultured human myometrial cells from term pregnant patients were challenged with compounds as indicated and phospho-ERK (Thr202/Tyr204) determined. Dose response of agonists at 5 mins for ERK activation. EC_{50} values were -6.93 ± 0.34 (118nM) and -5.10 ± 0.32 (8.03µM) for oxytocin and atosiban respectively. Data are mean ± s.e.m., n=3 (data were analysed by one-way ANOVA followed by Dunnett *post hoc* test comparing test compound to vehicle, and $P < 0.05$ considered significant, * $P < 0.05$, ** $P < 0.01$). (**b**) Atosiban and oxytocin response time course. Data are mean ± s.e.m., n=3 (data were analysed by two-way ANOVA followed by Dunnett *post hoc* test comparing test compound time series to vehicle, and $P < 0.05$ considered significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). (**c**) As (**b**) but cultured cells were pre-treated for 18 hours with 100 ng/ml PTX. Data are mean ± s.e.m., n=3.

Figure 9.

Human myometrial strips were challenged with various OTR ligands for times indicated before homogenization. Expression of COX-2 (**a**) and PKA (**b**) in homogenates and concentrations of PGE_2 (**c**) and $PGF_{2\alpha}$ (**d**) in organ bath supernatant were determined. Data are mean ± s.e.m., n=3 (data were analysed by two-way ANOVA followed by Dunnett *post hoc* test comparing test compound time series to vehicle, and $P < 0.05$ considered significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Figure 10.

Human myometrial strips were treated overnight with 100ng/ml PTX to inhibit $G_{\alpha i}$, for 1hr with 10μM U0126 to inhibit ERK signalling, or 10μM rofecoxib to inhibit COX-2 before challenge with either oxytocin or atosiban as indicated. PGE_2 (**a**) and $PGF_{2\alpha}$ (**b**) within organ bath supernatant and COX-2 levels in tissue homogenates (**c**) were determined. Data are mean ± s.e.m, n=3 (data were analysed by one-way ANOVA with atosiban and oxytocin analysed separately followed by Dunnett *post hoc* test comparing inhibitor compound to oxytocin or atosiban alone, and $P < 0.05$ considered significant, * $P < 0.05$, ** $P < 0.01$.)

.

Figure 11.

Membranes prepared from cultured human myometrial cells were stimulated with various compounds for 2 min in the presence of [^{35}S]-GTPγS and GDP. Activated membranes were incubated with antisera targeting $G\alpha_i$ (**a**), $G\alpha_{i/o}$ (**b**) and $G\alpha_s$ (**c**) G-proteins and with protein G-Sepharose beads. NSB was determined by inclusion of 10μM unlabeled GTPγS. Data are mean ± s.e.m., n=4 (data were analysed by one-way ANOVA followed by Dunnett *post hoc* test comparing test compound to vehicle, and $P < 0.05$ considered significant, * $P < 0.05$, ** $P < 0.01$).

Figure 12.

Schematic representation of the ligands of this study and their downstream signaling pathways in myometrium.

Abbreviations: AC: Adenylate Cyclase, ATP: Adenosine Trisphosphate, Ca^{2+}: Calcium, cAMP: cyclic Adenosine Monophosphate, COX-2: Cyclo-oxygenase 2, DAG: Diacylglycerol, ERK 1/2: Extracellular Regulated Kinase 1/2, IP_3: Inositol trisphosphate, MAP Kinase: Mitogen Activated Kinase, PIP_2: Phosphatidylinositol 4,5-bisphosphate, PGE_2: Prostaglandin E_2, $PGF_{2\alpha}$: Prostaglandin $F_{2\alpha}$, PKA: Protein Kinase A, PKC: Protein Kinase C, PLC: Phospholipase C, PTX: Pertussis Toxin.

Reference:

1. Blencowe H, Cousens S, Chou D, Oestergaard M, Say L, Moller AB, Kinney M, Lawn J, Born Too Soon Preterm Birth Action G: **Born too soon: the global epidemiology of 15 million preterm births**. *Reprod Health* 2013, **10 Suppl 1**:S2.
2. Goldenberg RL, Culhane JF, Iams JD, Romero R: **Epidemiology and causes of preterm birth**. *Lancet* 2008, **371**:75-84.
3. **Estimates of neonatal morbidities and disabilities at regional and global levels for 2010: introduction, methods overview, and relevant findings from the Global Burden of Disease study**. 2013, **74 Suppl 1**:4-16.
4. Nicholson WK, Frick KD, Powe NR: **Economic burden of hospitalizations for preterm labor in the United States**. *Obstet Gynecol* 2000, **96**:95-101.
5. Lamont RF, Jorgensen JS: **Safety and Efficacy of Tocolytics for the Treatment of Spontaneous Preterm Labour**. *Curr Pharm Des* 2019, **25**:577-592.
6. Manning M, Misicka A, Olma A, Bankowski K, Stoev S, Chini B, Durroux T, Mouillac B, Corbani M, Guillon G: **Oxytocin and vasopressin agonists and antagonists as research tools and potential therapeutics**. *J Neuroendocrinol* 2012, **24**:609-628.
7. Blanks AM, Thornton S: **The role of oxytocin in parturition**. *BJOG* 2003, **110 Suppl 20**:46-51.
8. Sanborn BM, Dodge K, Monga M, Qian A, Wang W, Yue C: **Molecular mechanisms regulating the effects of oxytocin on myometrial intracellular calcium**. *Adv Exp Med Biol* 1998, **449**:277-286.
9. Kim SH, MacIntyre DA, Firmino Da Silva M, Blanks AM, Lee YS, Thornton S, Bennett PR, Terzidou V: **Oxytocin activates NF-kappaB-mediated inflammatory pathways in human gestational tissues**. *Mol Cell Endocrinol* 2015, **403**:64-77.
10. Kim SH, MacIntyre DA, Hanyaloglu AC, Blanks AM, Thornton S, Bennett PR, Terzidou V: **The oxytocin receptor antagonist, Atosiban, activates pro-inflammatory pathways in human amnion via G(alphai) signalling**. *Mol Cell Endocrinol* 2016, **420**:11-23.
11. Liddle J, Allen MJ, Borthwick AD, Brooks DP, Davies DE, Edwards RM, Exall AM, Hamlett C, Irving WR, Mason AM, et al.: **The discovery of GSK221149A: a potent and selective oxytocin antagonist**. *Bioorg Med Chem Lett* 2008, **18**:90-94.
12. McCafferty GP, Pullen MA, Wu C, Edwards RM, Allen MJ, Woollard PM, Borthwick AD, Liddle J, Hickey DM, Brooks DP, et al.: **Use of a novel and highly selective oxytocin receptor antagonist to characterize uterine contractions in the rat**. *Am J Physiol Regul Integr Comp Physiol* 2007, **293**:R299-305.
13. Aye I, Moraitis AA, Stanislaus D, Charnock-Jones DS, Smith GCS: **Retosiban Prevents Stretch-Induced Human Myometrial Contractility and Delays Labor in Cynomolgus Monkeys**. *J Clin Endocrinol Metab* 2018, **103**:1056-1067.
14. Thornton S, Miller H, Valenzuela G, Snidow J, Stier B, Fossler MJ, Montague TH, Powell M, Beach KJ: **Treatment of spontaneous preterm labour with retosiban: a phase 2 proof-of-concept study**. *Br J Clin Pharmacol* 2015, **80**:740-749.
15. Akam EC, Challiss RA, Nahorski SR: **G(q/11) and G(i/o) activation profiles in CHO cells expressing human muscarinic acetylcholine receptors: dependence on agonist as well as receptor-subtype**. *Br J Pharmacol* 2001, **132**:950-958.
16. Burford NT, Tolbert LM, Sadee W: **Specific G protein activation and mu-opioid receptor internalization caused by morphine, DAMGO and endomorphin I**. *Eur J Pharmacol* 1998, **342**:123-126.

17. Friedman E, Butkerait P, Wang HY: **Analysis of receptor-stimulated and basal guanine nucleotide binding to membrane G proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis**. *Anal Biochem* 1993, **214**:171-178.
18. Wang HY, Undie AS, Friedman E: **Evidence for the coupling of Gq protein to D1-like dopamine sites in rat striatum: possible role in dopamine-mediated inositol phosphate formation**. *Mol Pharmacol* 1995, **48**:988-994.
19. Bradford MM: **A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding**. *Anal Biochem* 1976, **72**:248-254.
20. Fuchs AR, Fuchs F, Husslein P, Soloff MS: **Oxytocin receptors in the human uterus during pregnancy and parturition**. *Am J Obstet Gynecol* 1984, **150**:734-741.
21. Fuchs AR, Fuchs F, Husslein P, Soloff MS, Fernstrom MJ: **Oxytocin receptors and human parturition: a dual role for oxytocin in the initiation of labor**. *Science* 1982, **215**:1396-1398.
22. Clement P, Bernabe J, Compagnie S, Alexandre L, McCallum S, Giuliano F: **Inhibition of ejaculation by the non-peptide oxytocin receptor antagonist GSK557296: a multi-level site of action**. *Br J Pharmacol* 2013, **169**:1477-1485.
23. Borthwick AD, Liddle J, Davies DE, Exall AM, Hamlett C, Hickey DM, Mason AM, Smith IE, Nerozzi F, Peace S, et al.: **Pyridyl-2,5-diketopiperazines as potent, selective, and orally bioavailable oxytocin antagonists: synthesis, pharmacokinetics, and in vivo potency**. *J Med Chem* 2012, **55**:783-796.
24. Matsubara T, Moskowitz MA, Byun B: **CP-93,129, a potent and selective 5-HT1B receptor agonist blocks neurogenic plasma extravasation within rat but not guinea-pig dura mater**. *Br J Pharmacol* 1991, **104**:3-4.
25. Brighton PJ, Marczylo TH, Rana S, Konje JC, Willets JM: **Characterization of the endocannabinoid system, CB(1) receptor signalling and desensitization in human myometrium**. *Br J Pharmacol* 2011, **164**:1479-1494.
26. Ball DI, Brittain RT, Coleman RA, Denyer LH, Jack D, Johnson M, Lunts LH, Nials AT, Sheldrick KE, Skidmore IF: **Salmeterol, a novel, long-acting beta 2-adrenoceptor agonist: characterization of pharmacological activity in vitro and in vivo**. *Br J Pharmacol* 1991, **104**:665-671.
27. Blanks AM, Shmygol A, Thornton S: **Regulation of oxytocin receptors and oxytocin receptor signaling**. *Semin Reprod Med* 2007, **25**:52-59.