WARWICK
THE UNIVERSITY OF WARWICK

Manuscript version: Author's Accepted Manuscript
The version presented in WRAP is the author's accepted manuscript and may differ from the published version or Version of Record.

Persistent WRAP URL:
http://wrap.warwick.ac.uk/132459

How to cite:
Please refer to published version for the most recent bibliographic citation information. If a published version is known of, the repository item page linked to above, will contain details on accessing it.

Copyright and reuse:
The Warwick Research Archive Portal (WRAP) makes this work by researchers of the University of Warwick available open access under the following conditions.

Copyright © and all moral rights to the version of the paper presented here belong to the individual author(s) and/or other copyright owners. To the extent reasonable and practicable the material made available in WRAP has been checked for eligibility before being made available.

Copies of full items can be used for personal research or study, educational, or not-for-profit purposes without prior permission or charge. Provided that the authors, title and full bibliographic details are credited, a hyperlink and/or URL is given for the original metadata page and the content is not changed in any way.

Publisher's statement:
Please refer to the repository item page, publisher's statement section, for further information.

For more information, please contact the WRAP Team at: wrap@warwick.ac.uk.

1 **Functionally selective inhibition of the oxytocin receptor by retosiban in human**
2 **myometrial smooth muscle**
3
4 Paul J Brighton[1], Michael J. Fossler[2], Siobhan Quenby[1,3], Andrew M Blanks[1,4]
5
6 1: Cell and Developmental Biology, Division of Biomedical Sciences, Warwick Medical
7 School, University of Warwick. CV2 2DX
8 2: Senior Director, Clinical Pharmacology Modeling and Simulation, GlaxoSmithKline, Upper
9 Merion West, King of Prussia, PA.
10 Current address: Vice President, Clinical Development and Quantitative Sciences, Trevena
11 Inc., Chesterbrook, PA.
12 3: Maternity Directorate, University Hospital Coventry and Warwickshire, Coventry. CV2
13 2DX
14
15 4: **Address correspondence to:** Andrew M. Blanks, Cell and Developmental Biology,
16 Division of Biomedical Sciences, Warwick Medical School, Coventry CV2 2DX, UK. P: +44-
17 2476968703 F: +44-2476968653; Email:andrew.blanks@warwick.ac.uk
18
19 **Acknowledgments:**
20 We thank the patients and midwives of the Biomedical Research Unit at University Hospital
21 Coventry and Warwickshire for the collection and curation of myometrial samples.
22 **Funding:**
23 This work was funded by a project grant to A.M.B, S.Q. and P.J.B. from GlaxoSmithKline
24 **Conflict of interest:**
25 A.M.B. has undertaken scientific consultancy work for GlaxoSmithKline and Ferring
26 pharmaceuticals for the development of oxytocin receptor antagonists for the treatment of
27 preterm birth.
28 **Author Contributions:**
29 A.M.B. conceived of the project, acquired the grant, analysed data and co-wrote the
30 manuscript. P.J.B. undertook all experimental work, analysed data and co-wrote the
31 manuscript. S.Q. acquired and curated patient samples and edited the manuscript. M.J.F. co-
32 conceived the project, analysed data and edited the manuscript.
33

34 **Abstract**
35 **Context:** Novel small molecule inhibitors of the oxytocin receptor (OTR) may have distinct
36 pharmacology and mode of action when compared to first generation oxytocin antagonists
37 when used for the prevention of preterm birth.
38 **Objective:** To determine the mechanism of action of small molecule OTR antagonists
39 retosiban and epelsiban in comparison to the currently used peptide-based compound atosiban.
40 **Design:** Human myometrial samples were obtained at cesarean section and subjected to
41 pharmacological manipulations to establish the effect of antagonist binding to OTR on
42 downstream signaling.
43 **Results:** Retosiban antagonism of oxytocin action in human myometrium was potent, rapid
44 and reversible. Inhibition of inositol 1,4,5-trisphosphate (IP3) production followed single site
45 competitive binding kinetics for epelsiban, retosiban and atosiban. Retosiban inhibited basal
46 production of IP3 in the absence of oxytocin. Oxytocin and atosiban, but not retosiban inhibited
47 forskolin and calcitonin stimulated cAMP production. Inhibition of cAMP was reversed by
48 pertussis toxin. Oxytocin and atosiban, but not retosiban and epelsiban, stimulated ERK1/2
49 activity in a time a concentration dependent manner. Oxytocin and atosiban stimulated cyclo
50 oxygenase 2 (COX2) activity and subsequent production of prostaglandin E_2 and $F_{2\alpha}$.
51 Prostaglandin production was inhibited by rofecoxib, pertussin toxin, and ERK inhibitor
52 U0126. Oxytocin but not retosiban or atosiban stimulated coupling of the OTR to $G\alpha_q$ G-
53 proteins. Oxytocin and atosiban but not retosiban stimulated coupling of the OTR to $G\alpha_i$ G-
54 proteins.
55 **Conclusions:**
56 Retosiban and epelsiban demonstrate distinct pharmacology when compared to atosiban in
57 human myometrial smooth muscle. Atosiban displays agonist activity at micromolar
58 concentrations leading to stimulation of prostaglandin production.
59

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

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

126 **Radioligand binding**

127 Tissue preparation: Frozen tissue was ground to a fine powder using a pestle and mortar and
128 homogenised in ice-cold radioligand binding buffer (composition (mM): Tris-HCl; 50, EDTA;
129 2.5, and $MgSO_4$; 5, pH 7.4 (with KOH)). Homogenates were cleared (1000 rpm, 10 min 4°C),
130 pelleted (15000 rpm, 15 min, 4°C) and resuspended in radioligand binding buffer where protein
131 was adjusted to 1 mg/ml. Radioligand binding assay: Assays were performed in 500 µl volumes
132 of radioligand binding buffer containing ~100 µg membranes and [^{3}H]-Oxytocin (SA: 50
133 µCi/mmol) (Perkin Elmer. Massachusetts, U.S.A.) at concentrations ranging from 0.001-50
134 nM. Non-specific binding was determined by the inclusion of 100 nM unlabeled oxytocin.
135 Assays were equilibrated at 30°C for 2h before harvest through 0.5% polyethylenimine pre-
136 soaked Whatman GF/B filters. Recovered radiation was determined by standard liquid
137 scintillation counting. Data analysis: Specific binding was determined as total binding less non-
138 specific binding. Data were fitted using GraphPad Prism (v5.0) and the B_{max} and K_D values
139 obtained.

140 **Measurement of cAMP**

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

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

154 **Measurement of phosphorylated ERK (pERK) in cells**

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

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

167 Muscle strips approximately 5×2×2 mm were mounted horizontally in a DMT Flatbed muscle
168 strip myograph system (DMT, Hinnerup, Denmark). Strips were held under 20 mN tension in
169 m-KHB at 37°C wherein spontaneous contractions occurred. Drugs were added directly into
170 the organ bath as per protocol requirements. Tissue was snap frozen by rapid removal from
171 organ baths and immediate submersion into LN_2. Concurrently, aliquots of the supernatant
172 were collected for assessment of prostaglandin production. Where necessary, tissue biopsies
173 were treated with 100 ng/ml PTX overnight at 4°C to inhibit $G\alpha_i$ signaling, or 1h pre-treatment
174 with 10 µM U0126 to inhibit ERK signaling or 10 µM rofecoxib to inhibit COX-2. Preparation
175 of cell lysates: Frozen tissue was diced into small pieces before homogenisation in 1 ml RIPA
176 buffer containing protease (Pierce™ tablets) and phosphatase (phosphatase inhibitor cocktail
177 2 (diluted 1:100), (Sigma, Poole, UK)) inhibitors. Homogenisation was achieved using a NEXT
178 Advanced BBY24M Bullet Blender Storm (Next Advanced, Averill Park, NY, USA) for 10min
179 at 4°C, and the NAVY ball bearing mix (refer to manufacturer's instructions). Lysates were
180 subject to clearance (500 rpm, 2 min, 4°C) before transfer to duplicate tubes for assay and
181 protein quantification via a modified Lowry assay (DC™ Protein Assay, Bio-Rad, CA,
182 U.S.A.). Quantitation of PGE_2 and $PGF_{2\alpha}$ in tissue supernatant was via competitive ELISA
183 exactly as per manufacturers' instructions. The PGE_2 assay (KGE0048) was obtained from
184 R&D Systems (Minneapolis, MN, USA), and the $PGF_{2\alpha}$ assay (516011) from Cayman

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

191 **[^{35}S]-guanosine 5'-O-[gamma-thio]triphosphate ([^{35}S]GTPγS) binding**

192 [^{35}S]-GTPγS is a non-hydrolyzable analog of GTP that stays bound to activated G-proteins.
193 Specific G-protein activation can therefore be detected by virtue of the radiolabel, and co-
194 immunoprecipitation with specific Gα antibodies. The [^{35}S]-GTPγS assay was performed
195 according to a modification of methodologies described previously[15-18] Membrane
196 preparation: Primary myometrial cultures and CHO-hOTR cells were grown to confluence in
197 175cm^2 flasks, lifted and pelleted via standard cell culture techniques. Pelleted cells were
198 homogenized in the presence of hypotonic lysis buffer (10 mM EDTA, 10 mM HEPES, pH
199 7.4) using a Coleman handheld homogenizer. The homogenate was pre-cleared by
200 centrifugation (500 g, 5 min, 4°C) and the membranes collected via centrifugation (36,000g,
201 30 min, 4°C). Membranes were resuspended in freezing buffer (10 mM HEPES, 0.1 mM
202 EDTA, pH 7.4) at 6 mg/ml protein and rapidly frozen in liquid nitrogen. Membranes were
203 stored at -80°C until used. Receptor activation: Frozen membrane aliquots were diluted to 1.5
204 mg/ml in assay buffer (composition: (mM): HEPES; 10, NaCl; 100, $MgCl_2$; 10, pH 7.4) and
205 75 µg membrane were added to 50 µl of assay buffer containing 1 nM [^{35}S]-GTPγS (1250 Ci
206 mmol^{-1}) and 1 µM GDP, with or without ligands as required, and incubated at 30°C for 2 min.
207 Non-specific binding was determined by the inclusion of 10 µM unlabeled GTPγS. Incubations
208 were terminated by 900 µl ice-cold assay buffer and transfer to ice. Cell membranes were
209 recovered from the reaction mixture by centrifugation (20,000g, 6 min, 4°C) and the
210 supernatant removed by aspiration. Membrane pellets were solubilized by the addition of 50
211 µl ice-cold solubilization buffer (composition (mM): Tris/HCl; 100, NaCl; 200, EDTA; 1 and
212 1.25% (v:v) Igepal CA 630, pH 7.4) containing 0.2% (w:v) SDS and vortex-mixing. Once the
213 protein was completely solubilized, an equal volume of solubilization buffer without SDS was
214 added. Immuno-detection of G-Proteins: The solubilized protein was pre-cleared with normal
215 rabbit serum (1:100 dilution) and 30 µl of Protein-G sepharose beads (protein-G sepharose
216 bead suspension 30% w:v in TE buffer (10 mM Tris/HCl, 10 mM EDTA, pH 8.0)) for 60 min
217 at 4°C. The protein-G beads and any insoluble material were collected by centrifugation

218 (20,000g, 6 min, 4°C) then 100 µl of the supernatant was transferred to a fresh tube containing
219 G-protein antiserum (1:100 dilution). Samples were vortex-mixed and rotated overnight at 4°C.
220 To each sample tube was added 70 µl of Protein G-sepharose bead suspension and the samples
221 vortex-mixed and rotated for 90 min at 4°C. Protein G-sepharose beads were pelleted at 20,000
222 g and supernatant removed. Beads were washed and pelleted three times with 500 µl
223 solubilization buffer (less SDS) before re-suspension in scintillation cocktail where [^{35}S]-
224 GTPγS was determined by standard liquid scintillation counting methods. Data analysis: CPM
225 values were expressed as a % increase over basal (unstimulated).

226 **IP-One assay**

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

239 **Measurement of IP_3 in myometrial strips**

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

252 using the [^{3}H]-IP_3 Radioreceptor assay kit (PerkinElmer Life Inc, Boston, MA) as per the
253 manufacturer's instructions. All dilutions were considered and IP_3 was calculated in pmol/mg
254 protein.
255 Protein determination:
256 Unless otherwise stated, protein content was assessed by the Bradford method [19].
257 **Statistical analysis**
258 Contractility studies:
259 Experiments were repeated on biopsies obtained from different women where n represents the
260 number of biological replicates. For figures 1 and 2 data were analysed by one-way ANOVA
261 followed by Dunnett *post hoc* test comparing test compound to vehicle, and $P < 0.05$
262 considered significant. For figure 3 data were analysed by mixed model ANOVA with
263 antagonist as the between measures variable followed by Dunnett *post hoc* test comparing test
264 antagonist concentration to vehicle, and $P < 0.05$ considered significant. P values were
265 graphically represented as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. For figure 4 data were
266 analysed by paired T-Test comparing observations before and after retosiban treatment, and P
267 < 0.05 considered significant.
268 IP1 assay:
269 Experiments were repeated on CHO-hOTR cells where n represents the number of technical
270 replicates. For figure 5 data were analysed by mixed model ANOVA with antagonist as the
271 between measures variable followed by Dunnett *post hoc* test comparing test antagonist
272 concentration to vehicle, and $P < 0.05$ considered significant. P values were graphically
273 represented as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. IP-1 levels were interpolated from a 4-
274 parameter fit of known standards and plotted graphically for calculations of EC_{50} values using
275 GraphPad Prism (v5.0). For antagonist potency, pA_2 values were determined from the intercept
276 when y=0 using the Schild equation (Log (EC_{50} ratio -1) vs. Log [antagonist]).
277 IP_3 assay:
278 Experiments were repeated on myometrial membrane preparations from different women
279 where n represents the number of biological replicates. For figure 6 data were analysed by one-
280 way ANOVA followed by Dunnett *post hoc* test comparing test compound to vehicle, and $P <$
281 0.05 considered significant. Refractory period and peak contraction were analysed separately.
282 P values were graphically represented as * $P < 0.05$, ** $P < 0.01$.
283 cAMP assay:
284 Experiments were repeated on CHO-hOTR cells where n represents the number of technical
285 replicates. For figure 7 data were analysed by two-way ANOVA followed by Dunnett *post hoc*

286 test comparing test compound time series to vehicle, and $P < 0.05$ considered significant. P
287 values were graphically represented as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
288 ERK assay:
289 Experiments were repeated on primary human myometrial cultures derived from different
290 patient biopsies where n represents the number of biological replicates. For figure 8 data were
291 analysed by two-way ANOVA followed by Dunnett *post hoc* test comparing test compound
292 time series to vehicle, and $P < 0.05$ considered significant. P values were graphically
293 represented as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
294 COX-2, PKA, PGE_2 and $PGF_{2\alpha}$ assay:
295 Experiments were repeated on biopsies obtained from different women where n represents the
296 number of biological replicates. For figure 9 data were analysed by two-way ANOVA
297 followed by Dunnett *post hoc* test comparing test compound time series to vehicle, and $P <$
298 0.05 considered significant. P values were graphically represented as * $P < 0.05$, ** $P < 0.01$.
299 For figure 10 data were analysed by one-way ANOVA with atosiban and oxytocin analysed
300 separately followed by Dunnett *post hoc* test comparing inhibitor compound to oxytocin or
301 atosiban alone, and $P < 0.05$ considered significant. P values were graphically represented as
302 * $P < 0.05$, ** $P < 0.01$.
303 G-protein coupling assay:
304 Experiments were repeated on membrane preparations from primary human myometrial
305 cultures derived from different patient biopsies where n represents the number of biological
306 replicates. For figure 11 data were analysed by one-way ANOVA followed by Dunnett *post*
307 *hoc* test comparing test compound to vehicle, and $P < 0.05$ considered significant. P values
308 were graphically represented as * $P < 0.05$, ** $P < 0.01$.

309 **Results**

310 Prior to detailed studies of the OTR signaling in myometrial strips, we first characterized the
311 basic physiological responses to oxytocin. The addition of increasing concentrations of
312 oxytocin to human myometrial smooth-muscle strips evoked a concentration-dependent
313 increase in the magnitude, integral and frequency of spontaneous contractions (**Fig. 1a and b**),
314 whereas no changes were observed by addition of vehicle control (**Fig. 1b**). Concentration-
315 response analysis (**Fig. 1b**) revealed EC_{50} values of: peak -8.224 ± 0.46 (5.9 nM), integral -
316 8.053 ± 0.13 (8.8 nM), and frequency -8.229 ± 0.16 (5.0 nM). In radioligand binding studies,
317 membranes were prepared from whole muscle biopsies and saturated with [^{3}H]-oxytocin to
318 permit the quantification of OTR expression. The binding of [^{3}H]-oxytocin to membranes
319 prepared from biopsies was saturable. Specific binding, as determined by inclusion of 100nM
320 oxytocin represented approximately 40% of the total binding at a K_D concentration of [^{3}H]-
321 oxytocin. Analysis of saturation binding curves indicated a B_{max} value of 8337 ± 931 fmol/mg
322 protein, and a K_D value of 24.15 ± 6.5nM for oxytocin (**Fig. 1c**).

323 The saturation studies confirmed previous studies that the OTR reaches high concentrations in
324 the myometrium at term [20,21]. Under such conditions it is feasible that unliganded receptor
325 contributes to downstream signaling. Taken together with previous studies suggesting that
326 retosiban may be an inverse agonist (defined as a ligand that can reduce constitutive receptor
327 activity)[13], we sought to investigate whether retosiban could reduce spontaneous
328 contractions in the absence of oxytocin. The addition of retosiban (**Fig. 2a**) or atosiban (**Fig.**
329 **2b**) did not significantly reduce the mean of magnitude, activity integral, or frequency of
330 contractions in spontaneously contracting strips. In competition assays using oxytocin as
331 agonist, however, the presence of increasing concentrations of retosiban evoked a right-ward
332 shift in oxytocin concentration-response curve for peak (**Fig. 3a**), integral (**Fig. 3b**) and
333 frequency (**Fig. 3c**). Similar observations were made with atosiban (**Fig. 3d-f**). EC_{50} values
334 observed are displayed in table 1.

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

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

343 their mechanism of downstream signaling and potency. To confirm that any ligand bias was
344 not simply a myometrial cell phenomenon we first tested the effect of the OTR antagonists on
345 a Chinese Hamster Ovary cell line stably expressing the human oxytocin receptor. Oxytocin
346 generated concentration-dependent increases in IP-1 accumulation (**Fig. 5a-c**), a stable
347 metabolite of IP_3. Maximal responses were achieved at 100μM oxytocin with an EC_{50} value of
348 -6.48±0.17 (332nM). The presence of increasing concentrations of retosiban (**Fig. 5a**), atosiban
349 (**Fig. 5b**), and epelsiban an additional potent small molecule inhibitor of the OTR [22,23]
350 provoked a right-ward curve shift (**Fig. 5c**), whereby increasing concentrations of oxytocin
351 were required to illicit the same responses. pA_2 values (the dose of antagonist needed to shift
352 the agonist dose-response curve 2-fold to the right) were obtained using the Schild equation
353 (**Fig. 5d**). Of the 3 antagonists, epelsiban was most potent with a pA_2 value of -8.537, followed
354 by retosiban -8.045 and finally atosiban -6.159. The data would indicate an increase in potency
355 of inhibition of IP_3 generation as approximately 77-fold for retosiban and 240-fold for
356 epelsiban when compared to atosiban. Further analysis of Schild plots revealed slopes of 1.017,
357 1.069 and 1.042 for epelsiban, retosiban and atosiban, respectively. pA_2 and slope values are
358 detailed in Table 2.
359 To investigate IP_3 production in intact tissue, uterine strips were treated with various
360 compounds, and snap frozen either mid-way between or at the peak of contractions and IP_3
361 content determined. Initial comparisons of contraction versus inter-contraction interval
362 demonstrated IP_3 levels were essentially the same irrespective of contractions (**Fig. 6a**). By
363 contrast 100nM oxytocin markedly increased IP_3 levels (**Fig. 6a**), whilst IP_3 was significantly
364 reduced in the presence of 1μM retosiban (**Fig. 6a**). To investigate and confirm this effect
365 further we increased the number of observations during peak contraction whereby retosiban
366 again decreased IP_3 when compared to vehicle control whereas IP_3 levels in 10μM atosiban-
367 treated strips decreased but did not reach significance (**Fig.6b**).
368 To further investigate downstream signaling and potential ligand bias at the OTR we
369 challenged CHO-*h*OTR cells with either oxytocin, atosiban, retosiban or epelsiban and
370 measured cAMP accumulation. None of the ligands elevated levels of cAMP (**Fig. 7a**), whereas
371 addition of either forskolin or calcitonin, a selective $G\alpha_s$-coupled receptor agonist, caused a
372 robust and saturable elevation of cAMP accumulation. These data suggest that the OTR ligands
373 do not signal through $G\alpha_s$ G-proteins in these cells.
374 Both oxytocin and atosiban reduced calcitonin (**Fig. 7b**) and forskolin mediated cAMP
375 production (**Fig. 7c**), providing evidence of $G\alpha_i$-coupling. The potent and selective 5-HT_{1B}
376 ($G\alpha_i$-coupled) agonist CP93129 [24] evoked similar changes. Importantly, neither retosiban

377 nor epelsiban had any effect on cAMP. The oxytocin and atosiban mediated inhibition of
378 cAMP were PTX sensitive demonstrating $G\alpha_i$ involvement (**Fig. 7d**).
379 Stimulation of $G\alpha_i$ signaling in myometrial cells has the potential to activate ERK [25]. We
380 therefore tested whether challenge of cultured myometrial cells taken at term cesarean section
381 with either oxytocin or atosiban evoked an increase in ERK phosphorylation. Application of
382 either oxytocin or atosiban increased ERK activity in a concentration-dependent manner (**Fig.
383 8a**) and generated EC_{50} values of -6.93 ± 0.34 (118nM) and -5.10 ± 0.32 (8.03μM) for oxytocin
384 and atosiban respectively that peaked at ~5 min and subsequently declined (**Fig. 8b**). Cells did
385 not respond to either controls, retosiban or epelsiban at equivalent receptor occupancy.
386 Oxytocin and atosiban-mediated responses were inhibited by preincubation with pertussis
387 toxin (PTX) (**Fig. 8c**).
388 Increases in ERK activity in myometrial smooth muscle are associated with increased
389 production of prostaglandins. We observed an increase in COX-2 (**Fig. 9a**) expression, and
390 PGE_2 (**Fig. 9c**) and $PGF_{2\alpha}$ (**Fig. 9d**) secretion on challenge of spontaneously contracting
391 myometrial strips with oxytocin and atosiban, but not retosiban or epelsiban. COX-2
392 expression increased rapidly, but PGE_2 and $PGF_{2\alpha}$ secretion was absent until 2hr where a 3 to
393 4-fold increase was observed. The magnitude of responses was similar for both oxytocin and
394 atosiban. No increases in protein kinase A (PKA) activity were detected (except with the
395 positive control forskolin), and no reduction in basal PKA levels were observed (**Fig. 9b**).
396 PGE_2 (**Fig. 10a**) and $PGF_{2\alpha}$ (**Fig. 10b**) secretion was inhibited by PTX ($G\alpha_i$ inhibition), U0126
397 (ERK inhibition) and rofecoxib (COX-2 inhibition). COX-2 expression was inhibited by PTX
398 and U0126 (**Fig. 10c**).
399 The downstream signaling analysis of the ligands suggested that contrary to atosiban being a
400 neutral antagonist, it is in fact, an agonist at $G\alpha_i$ at micromolar concentrations. To investigate
401 coupling of the OTR to specific Gα-subunits we utilised the [^{35}S]-GTPγS immunoprecipitation
402 assay in membrane preparations from cultured myometrial cells taken from patients at term.
403 Oxytocin increased [^{35}S]-GTPγS binding to $G\alpha_{q/11}$ (**Fig. 11a**) G-proteins and both oxytocin and
404 atosiban increased binding of $G\alpha_i$ subunits (**Fig. 11b**). Consistent with cAMP data no binding
405 was observed following immunoprecipitation with $G\alpha_s$ G-proteins (**Fig. 11c**). Combined with
406 signaling analysis these data confirm that at micromolar concentrations atosiban couples OTR
407 to $G\alpha_i$ to elicit activation of ERK in human myometrial cells. Importantly, neither retosiban
408 nor epelsiban elicited any increases in [^{35}S]-GTPγS binding, signifying no agonist activity with
409 these compounds.

411 **Discussion**

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

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

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

439 The functional consequences of the ligand bias of atosiban *in vivo* are hard to interpret without
440 accurate tissue concentrations. Tissue concentrations that reach, or exceed, micromolar levels
441 are likely to elicit inflammation and release of prostaglandins some hours after treatment. Thus,
442 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

459 Figure Legends:

460

461 Figure 1.

462 (**a**) Representative trace showing phasic myometrial contractions in response to increasing
463 concentrations of oxytocin (0.01nM-100nM). (**b**) Oxytocin (red square) concentration-
464 response curves. Data were analysed to assess the peak responses (peak-minimum), the area
465 under the curve (integral) and the peak frequency (Hz) during the 10 min oxytocin stimulation.
466 Data were expressed as a percentage increase over basal, unstimulated contractions in the
467 presence of vehicle (black circle). Data revealed EC_{50} values of peak: -8.224 ± 0.46 (5.9nm),
468 integral: -8.053 ± 0.13 (8.8nm) and peak frequency: -8.229 ± 0.16 (5.0nm). Data are mean ±
469 s.e.m., n=3 (One-Way ANOVA and Dunnett's Multiple Comparison Test comparing oxytocin
470 treatment to vehicle control, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) (**c**) Saturation binding in
471 membranes prepared from myometrial biopsies incubated with varying concentrations of [^{3}H]-
472 Oxytocin (0.01pM-100nM) either in the presence (non-specific binding) or absence (total
473 binding) of 100nM unlabeled oxytocin. B_{max} and K_D were 8337 ± 931 fmol/mg protein, and
474 24.15 ± 6.5nM, respectively. Data are mean ± s.e.m., n=6

475

476 Figure 2.

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

482

483 Figure 3.

484 Antagonist/agonist competition assays. Full oxytocin concentration-response curves were
485 performed in the continued presence of either vehicle, or various concentrations of retosiban
486 (0.001µM - 1µM) (**a-c**) or atosiban (0.01µM - 10µM) (**d-f**). Oxytocin doses were added for 10
487 minutes and separated by a 20 minutes wash period during which the desired concentration of
488 retosiban was continually present. Data were analysed in 10 minute blocks to assess the peak
489 responses (peak-minimum) (***a and d***), the area under the curve (integral) (***b and e***) and the peak
490 frequency (Hz) (***c and f***). Data were expressed as a percentage increase of basal, unstimulated
491 contractions. Data are mean ± s.e.m., n=10 (data were analysed by mixed model ANOVA with
492 antagonist as the between measures variable followed by Dunnett *post hoc* test comparing test

493 antagonist concentration to vehicle, and *P* < 0.05 considered significant * *P* < 0.05, ** *P* <
494 0.01, *** *P* < 0.001). EC_{50} and E_{max} values obtained are shown in table 1.
495
496 Figure 4.
497 Reversibility of retosiban antagonism (**a**) Representative traces showing the immediately
498 reversible effects of 100nm oxytocin and 1µM retosiban on myometrial muscle strips. (**b**) Peak
499 responses (peak-minimum), the area under the curve (integral) and the peak frequency (Hz)
500 were expressed as a percentage increase of basal, unstimulated contractions. Colours and
501 symbols denote paired observations within a biological replicate. Data are mean ± s.e.m., n=3
502 (data were analysed by paired T-Test comparing observations before and after retosiban
503 treatment, and *P* < 0.05 considered significant).
504
505 Figure 5.
506 Oxytocin-mediated IP-1 accumulation in CHO-hOTR cells. Cells were stimulated for 1hr with
507 oxytocin in the presence of increasing concentrations of retosiban (**a**) atosiban (**b**) or epelsiban
508 (**c**). Data are mean ± s.e.m, n=3 (data were analysed by mixed model ANOVA with antagonist
509 as the between measures variable followed by Dunnett *post hoc* test comparing test antagonist
510 concentration to vehicle, and *P* < 0.05 considered significant, * *P* < 0.05, ** *P* < 0.01, *** *P* <
511 0.001). For Schild plot analysis of antagonist activity EC_{50} values were converted using Schild
512 equation (Log (EC_{50} ratio-1) vs. Log [antagonist]) and pA_2 values obtained where y=0 (**d**). pA_2
513 and slope analysis values observed are indicated in table 2.
514
515 Figure 6.
516 IP_3 levels in frozen muscle strips. (**a**) Tissues were frozen in LN_2 between contractions
517 (refractory period) or at the peak of contraction in the presence of vehicle, 1µM retosiban,
518 10µM atosiban or 100nM oxytocin. IP_3 levels in oxytocin-challenged muscle strips were
519 significantly greater when compared to vehicle. The addition of 1µM retosiban significantly
520 reduced IP_3 when compared to vehicle. mean ± s.e.m., n=5 (data were analysed by one-way
521 ANOVA followed by Dunnett *post hoc* test comparing test compound to vehicle, and *P* < 0.05
522 considered significant. Refractory period and peak contraction were analysed separately, * *P*
523 < 0.05, ** *P* < 0.01) (**b**) To confirm the observation that retosiban reduced IP_3 levels in
524 spontaneously contracting strips we repeated the experimental protocol in (**a**) with more
525 numbers, measuring IP_3 during the refractory period. Again, retosiban significantly reduced
526 IP_3. mean ± s.e.m., n=10.

527 Figure 7.
528 (**a**) CHO-*h*OTR cells were challenged with compounds for time points indicated and
529 lysed. Data are mean ± s.e.m., n=3. (**b**) CHO-hOTR cells were challenged simultaneously with
530 compound and 10nM calcitonin for time points indicated before lysis. Inhibition of cAMP by
531 oxytocin, atosiban and CP93129 (a potent and selective 5-HT1B receptor ($G_{\alpha i}$-coupled)
532 agonist) was significant when compared to calcitonin + vehicle. Data are mean ± s.e.m., n=3.
533 (**c**) CHO-hOTR cells were stimulated with compounds for time points indicated before FSK
534 challenge (10μM, 10min). Atosiban, oxytocin and CP93129 reduced FSK elevated cAMP
535 when compared to vehicle. Data are mean ± s.e.m., n=3 (data were analysed by two-way
536 ANOVA followed by Dunnett *post hoc* test comparing test compound time series to vehicle,
537 and $P < 0.05$ considered significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). (**d**) As (**c**) but
538 cells were treated with either 100ng/ml PTX or vehicle for 18hrs prior to stimulus. PTX pre-
539 treatment reversed the inhibition of cAMP production by oxytocin atosiban and CP93129.
540
541 Figure 8.
542 (**a**) Cultured human myometrial cells from term pregnant patients were challenged with
543 compounds as indicated and phospho-ERK (Thr202/Tyr204) determined. Dose response of
544 agonists at 5 mins for ERK activation. EC_{50} values were -6.93 ± 0.34 (118nM) and -5.10 ± 0.32
545 (8.03μM) for oxytocin and atosiban respectively. Data are mean ± s.e.m., n=3 (data were
546 analysed by one-way ANOVA followed by Dunnett *post hoc* test comparing test compound to
547 vehicle, and $P < 0.05$ considered significant, * $P < 0.05$, ** $P < 0.01$). (**b**) Atosiban and
548 oxytocin response time course. Data are mean ± s.e.m., n=3 (data were analysed by two-way
549 ANOVA followed by Dunnett *post hoc* test comparing test compound time series to vehicle,
550 and $P < 0.05$ considered significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). (**c**) As (**b**) but
551 cultured cells were pre-treated for 18 hours with 100 ng/ml PTX. Data are mean ± s.e.m., n=3.
552
553 Figure 9.
554 Human myometrial strips were challenged with various OTR ligands for times indicated before
555 homogenization. Expression of COX-2 (**a**) and PKA (**b**) in homogenates and concentrations of
556 PGE_2 (**c**) and $PGF_{2\alpha}$ (**d**) in organ bath supernatant were determined. Data are mean ± s.e.m.,
557 n=3 (data were analysed by two-way ANOVA followed by Dunnett *post hoc* test comparing
558 test compound time series to vehicle, and $P < 0.05$ considered significant, * $P < 0.05$, ** $P <$
559 0.01, *** $P < 0.001$).

560

561 Figure 10.

562 Human myometrial strips were treated overnight with 100ng/ml PTX to inhibit $G_{\alpha i}$, for 1hr
563 with 10μM U0126 to inhibit ERK signalling, or 10μM rofecoxib to inhibit COX-2 before
564 challenge with either oxytocin or atosiban as indicated. PGE_2 (**a**) and $PGF_{2\alpha}$ (**b**) within organ
565 bath supernatant and COX-2 levels in tissue homogenates (**c**) were determined. Data are mean
566 ± s.e.m, n=3 (data were analysed by one-way ANOVA with atosiban and oxytocin analysed
567 separately followed by Dunnett *post hoc* test comparing inhibitor compound to oxytocin or
568 atosiban alone, and $P < 0.05$ considered significant, * $P < 0.05$, ** $P < 0.01$.)
569 .
570

571 Figure 11.

572 Membranes prepared from cultured human myometrial cells were stimulated with various
573 compounds for 2 min in the presence of [^{35}S]-GTPγS and GDP. Activated membranes were
574 incubated with antisera targeting $G\alpha_i$ (**a**), $G\alpha_{i/o}$ (**b**) and $G\alpha_s$ (**c**) G-proteins and with protein G-
575 Sepharose beads. NSB was determined by inclusion of 10μM unlabeled GTPγS. Data are mean
576 ± s.e.m., n=4 (data were analysed by one-way ANOVA followed by Dunnett *post hoc* test
577 comparing test compound to vehicle, and $P < 0.05$ considered significant, * $P < 0.05$, ** $P <$
578 0.01).
579

580 Figure 12.

581 Schematic representation of the ligands of this study and their downstream signaling pathways
582 in myometrium.
583

584 Abbreviations: AC: Adenylate Cyclase, ATP: Adenosine Trisphosphate, Ca^{2+}: Calcium,
585 cAMP: cyclic Adenosine Monophosphate, COX-2: Cyclo-oxygenase 2, DAG: Diacylglycerol,
586 ERK 1/2: Extracellular Regulated Kinase 1/2, IP_3: Inositol trisphosphate, MAP Kinase:
587 Mitogen Activated Kinase, PIP_2: Phosphatidylinositol 4,5-bisphosphate, PGE_2: Prostaglandin
588 E_2, $PGF_{2\alpha}$: Prostaglandin $F_{2\alpha}$, PKA: Protein Kinase A, PKC: Protein Kinase C, PLC:
589 Phospholipase C, PTX: Pertussis Toxin.
590
591
592

593
594
595
596

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.

643 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.
646 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.
649 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.
652 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.
654 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.
657 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.
660 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.
664 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.
667 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.
670 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.
674 27. Blanks AM, Shmygol A, Thornton S: **Regulation of oxytocin receptors and oxytocin receptor signaling**. *Semin Reprod Med* 2007, **25**:52-59.
676