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Drug Delivery to the Human and Mouse Uterus using Immunoliposomes Targeted to the Oxytocin Receptor

Jonathan W. PAUL[1,3,†] (PhD), Susan HUA,[2,3†] (PhD), Marina ILICIC[1,3] (BSc (Hons)), Jorge M. TOLOSA[1,3] (PhD), Trent BUTLER[1,3] (BSc (Hons)) and Roger SMITH[1,3,4*] (MBBS, PhD)

[1] Mothers and Babies Research Centre, School of Medicine and Public Health, Faculty of Health and Medicine, University of Newcastle, Callaghan, NSW, Australia, 2308;

[2] School of Biomedical Sciences and Pharmacy, Faculty of Health and Medicine, University of Newcastle, Callaghan, NSW, Australia, 2308;

[3] Hunter Medical Research Institute, 1 Kookaburra Circuit, New Lambton Heights, NSW, Australia, 2305;

[4] John Hunter Hospital, New Lambton Heights, NSW, Australia, 2305

[†] These authors contributed equally to this work.

***Corresponding Author:** Roger Smith, Hunter Medical Research Institute, New Lambton Heights 2305, NSW, Australia. Phone: +61 4042 0337 ; Fax: +61 4042 0045 ; Email: Roger.Smith@newcastle.edu.au

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Oxytocin receptor-targeted immunoliposomes inhibit human myometrial contractions, localize to the uterus of pregnant mice, and can be used to reduce rates of LPS-induced preterm birth.

Short version of title:

A Targeted Drug Delivery System for the Uterus

Abstract

Background: The ability to provide safe and effective pharmacotherapy during obstetric complications, such as preterm labor or post-partum hemorrhage, is hampered by the systemic toxicity of therapeutic agents leading to adverse side effects in mother and fetus. Development of novel strategies to target tocolytic and uterotonic agents specifically to uterine myocytes would improve therapeutic efficacy while minimizing the risk of side effects. Ligand-targeted liposomes have emerged as a reliable and versatile platform for targeted drug delivery to specific cell types, tissues or organs.

Objective: Our objective was to develop a targeted drug delivery system for the uterus utilizing an immunoliposome platform targeting the oxytocin receptor.

Study Design: We conjugated liposomes to an antibody that recognizes an extracellular domain of the oxytocin receptor. We then examined the ability of oxytocin receptor-targeted liposomes to deliver contraction blocking (nifedipine, salbutamol and rolipram) or contraction enhancing (dofetilide) agents to strips of spontaneously contracting myometrial tissue *in vitro* (human and mouse). We evaluated the ability of oxytocin receptor-targeted liposomes to localize to uterine tissue *in vivo*, and assessed if targeted liposomes loaded with indomethacin were capable of preventing lipopolysaccharide-induced preterm birth in mice.

Results: Oxytocin receptor-targeted liposomes loaded with nifedipine, salbutamol or rolipram consistently abolished human myometrial contractions *in vitro*, while oxytocin receptor-targeted liposomes loaded with dofetilide increased contraction duration. Non-targeted control liposomes loaded with these agents had no effect. Similar results were observed in mouse uterine strips. Following *in vivo* administration to pregnant mice, oxytocin receptor-targeted liposomes localized specifically to the uterine horns and mammary tissue. Targeting increased localization to the uterus 7-fold. Localization was not detected in the maternal brain or fetus. Targeted and non-targeted liposomes also localized to the liver. Oxytocin receptor-targeted liposomes loaded with indomethacin were effective in reducing

rates of preterm birth in mice, whereas non-targeted liposomes loaded with indomethacin had no effect.

Conclusion: Our results demonstrate that oxytocin receptor-targeted liposomes can be used to either inhibit or enhance human uterine contractions *in vitro*. *In vivo*, the liposomes localize to the uterine tissue of pregnant mice and were effective in delivering agents for the prevention of inflammation induced preterm labor. The potential clinical advantage of targeted liposomal drug delivery to the myometrium is reduced dose and reduced toxicity to both mother and fetus.

Key words: contraction, drug delivery, human, immunoliposomes, labor, liposomes, mouse, myometrium, oxytocin receptor, preterm, birth, targeted, tocolysis, uterotonic.

Introduction

Complications arising from preterm birth (PTB) are the leading cause of death among children under 5 years of age, accounting for nearly 1 million deaths in 2013,[1] while postpartum hemorrhage (PPH) is the leading cause of maternal mortality worldwide, accounting for up to 27.1% of maternal deaths.[2] Given that both can arise from dysregulation of uterine contractility, the need exists for safe and effective clinical interventions capable of modifying myometrial contractions to improve treatment of women in preterm labor, to induce or facilitate labor and to prevent or treat PPH, without adverse off-target effects on either the mother or fetus.

When a woman presents with preterm labor attempts are often made to halt contractions by administering tocolytics that inhibit or block components of the contraction cascade. A recent study proposed that "The ideal tocolytic agent should be myometrium-specific, easy to administer, inexpensive, effective in preventing PTB and improve neonatal outcomes, with few maternal, fetal, and neonatal side effects, and without long-term adverse effects".[3] Standard therapy varies from country to country, but tocolysis may involve the administration of calcium channel blockers, such as nifedipine (NIF), or β_2-adrenergic receptor agonists, such as salbutamol (SAL), an oxytocin receptor antagonist, such as atosiban, or a prostaglandin (PG) synthetase inhibitor, such as indomethacin (IND).[4-9] Unfortunately, the systemic administration of these therapies and lack of specificity means that large doses need to be administered in order to achieve a therapeutic effect at the target tissue, the myometrium. Maternal side effects of β_2-adrenergic receptor agonists include tremors, heart palpitations and tachycardia, as well as myocardial ischemia and pulmonary oedema.[8,10-12] NIF has been associated with fewer side effects, however approximately 1% of women experience a severe side effect and a further 1% experience mild adverse side effects.[13] Atosiban is associated with the lowest side effect risk but the efficacy of this agent is disputed.[14,15] Usefulness of IND is limited by fetal side effects, such as premature closure of the ductus arteriosus.[16-18] Achieving targeted drug delivery to the myometrium would

reduce the quantity of drug required to achieve therapeutic efficacy, reduce the likelihood of maternal and fetal side effects, and would therefore represent a significant advancement for maternal-fetal medicine.[12,19-21]

Targeted liposomes have emerged as a platform for achieving the delivery of drugs to specific tissues. Liposomes are artificial vesicles that range in size from 50 – 1000 nm, and are comprised of one or more phospholipid bilayers.[22,23] Liposomes are able to encapsulate both lipophilic and/or hydrophilic drugs, and are non-toxic and biodegradable with minimal immunogenicity.[21,24,25] Liposomal encapsulation improves the pharmacokinetics of drugs, particularly if the liposome surface is PEGylated, which reduces uptake by the reticuloendothelial system and prolongs half-life.[26] This has led to the development of liposomal-based preparations of various agents, including doxorubicin, amphotericin B, daunorubicin, and verteporfin.[27] Ligand-targeted liposomes offer the potential for site-specific delivery of drugs to designated cell types or organs *in vivo* that selectively express specific cell surface cognate receptors.[26] Although many types of targeting molecules are available, such as peptides/proteins and carbohydrates, the coupling of antibodies to the liposome surface to create immunoliposomes has many advantages. One advantage of using antibodies is their stability and higher binding avidity because of the presence of dual binding sites.[26] For example, liposomes coated with antibodies to intercellular adhesion molecule-1 (ICAM-1) have been developed for the treatment inflammatory diseases.[28] Administration of ICAM-1-targeted immunoliposomes loaded with an analgesic agent demonstrated specific localization and therapeutic efficacy exclusively in peripheral inflammatory tissue. All control groups (free drug solution, empty non-targeted liposomes, drug-loaded non-targeted liposomes, and empty ICAM-1-targeted immunoliposomes) showed no significant therapeutic response.[29]

The aim of this study was to develop a means of targeting therapeutic agents to uterine myometrial tissue, in order to allow therapeutic modification of myometrial

contractions in obstetric settings, such as preterm labor, labor induction and PPH. The expression of the oxytocin receptor (OTR) is significantly upregulated in myometrial cells approaching term.[30,31] Here we report the development of OTR-targeted PEGylated immunoliposomes loaded with traditional tocolytics, such as NIF and SAL, as well as rolipram (ROL), a phosphodiesterase 4 (PDE4) inhibitor and potent inhibitor of myometrial contractions.[32-34] Moreover, we report enhancement of human myometrial contraction duration *in vitro* through liposomal delivery of dofetilide (DOF), a hERG channel blocker that increases myometrial contraction duration,[35,36] demonstrating that this delivery platform can be used to either inhibit or enhance contractions in human myometrial tissue. We demonstrate that intravenously administered OTR-targeted liposomes localize specifically to the uterine tissue of pregnant mice *in vivo*. Finally, using an inflammatory mouse model of preterm birth (lipopolysaccharide (LPS) administration), we show that OTR-targeted liposomes loaded with IND are effective in preventing preterm birth, while IND loaded non-targeted liposomes have no effect.

Materials and Methods

Myometrial Tissue acquisition

Human Studies: These studies were performed in Newcastle, New South Wales, Australia and were approved by the Hunter and New England Area Human Research Ethics Committee, adhering to guidelines of the University of Newcastle and John Hunter Hospital, Newcastle, Australia (02/06/12/3.13). All participants gave informed written consent. Collection of myometrial samples (5 × 5 × 10 mm) occurred from the lower uterine segment of term singleton pregnancies. All women were examined clinically, and those with signs of infection were excluded. Women were undergoing term elective cesarean delivery and were not-in-labor (NIL); the clinical indications for elective NIL cesarean delivery were previous

cesarean section or previous 3rd/4th degree tear. All participants ranged from 37 – 40 completed weeks of gestation. Following delivery of the placenta, all women immediately received 5 units of oxytocin (syntocinon) into an intravenous line, which was administered as standard care. Myometrial biopsies were excised 3 – 5 min after oxytocin administration, thus all samples were briefly exposed to oxytocin. After biopsy, myometrial samples were dissected from connective tissue and washed in ice-cold physiological saline.

Mouse *In Vitro* Studies: Mouse uterine horns were dissected from pregnant CD1 Swiss mice (8 – 10 weeks of age) at term gestation prior to the onset of labor (fetal gestation day 18). Mouse studies were approved by the University of Newcastle Animal Ethics Committee (A-2014-400 / A-2014-429). All mice were housed under SPF/PC2 conditions under a 12 h light day cycle and had food and water available *ad libitum.*

Liposome Manufacture

Liposomes containing NIF, SAL, ROL, DOF (each at approximately 4 mg/mL) or IND (approximately 5.5 mg/mL), as determined by high performance liquid chromatography (HPLC), were manufactured as previously outlined.[28] Liposomes were composed of 1,2-distearoyl-sn-glycero-2-phosphocholine (DSPC) and cholesterol in a molar ratio of 2:1, and contained 1,2-distearoyl-*sn*-glycero-3-phospho-ethanolamine-N-[maleimide (polyethylene glycol)-2000] (DSPE-PEG(2000) maleimide) at 1.5 mol percent of DSPC as a coupling lipid (Avanti Polar Lipids). The resulting multilamellar dispersions were reduced in size and lamellarity to approximately 200 nm in diameter by high-pressure extrusion. The activated liposome suspension was then mixed with thiolated polyclonal anti-OTR antibody (Abcam, Cat# ab115664), which was prepared by first conjugating 25 µg of OTR antibody with a heterobifunctional reagent N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) (Figure 1). The OTR antibody recognizes an extracellular domain of the human OTR. Non-targeted liposomes were coated with rabbit immunoglobulin G (IgG). All liposomes incorporated the

membrane stain 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) for fluorescent detection. Unconjugated antibody and non-encapsulated drug was removed by centrifugal filtration of the liposomes through a 100 kDa molecular weight filter (Amicon Ultra-15). Amicon Ultra-15 filters were washed with Milli-Q H_2O before 500 µL of liposome suspension was loaded into the filter reservoir. Liposomes were diluted with 5 mL of sterile 0.9% saline and centrifuged at 4000 x g until retentate volume was <250 µL. Liposomes were then resuspended in a futher 5 mL of 0.9% saline and centrifuged until retentate volume was <250 µL. Filtered liposomes were then collected, transferred to a fresh Eppendorf tube and redispersed to an original volume of 500 µL.

The size distribution of the liposomal dispersion was determined by dynamic laser light scattering (Zetasizer Nano S^{TM}, ATA Scientific). Encapsulation efficiency (EE%) was determined by disrupting the vesicles with ethanol and evaluating drug concentration using HPLC. Quantification of the amount of antibody associated with liposomes was determined using the CBQCA protein assay (ThermoFisher Scientific Inc. Watham, MA, USA), using bovine serum albumin for the preparation of the standard curve.

Myometrial contractility studies

Myometrial strips were set up as previously described.[37] Briefly, NIL human myometrial samples, or uterine horns obtained from pregnant CD1 Swiss mice, were dissected into strips (10 × 2 × 2 mm) and suspended in organ baths containing 30 mL physiological saline solution (PSS) containing (in mM) NaCl 120, KCl 5, $NaHCO_3$ 25, KH_2PO_4 1, $MgSO_4$ 1.2, $CaCl_2$ 2.5 and glucose 11, and continuously gassed with carbogen (95% O_2, 5% CO_2), at pH 7.4. Strips were connected to a Grass FT03C force transducer (Grass Instruments, Quincy, MA) and 1 g passive tension applied (1 g was calibrated to equal 1 V). PSS was replaced five times during the first hour, with strips re-tensioned to 1 g passive tension following each wash. Thereafter strips were maintained at 37°C until spontaneous rhythmic

contractions developed. Data were digitized using a MacLab/8E data-acquisition system and contraction status visualized in real time using Chart software (ADInstruments, NZ). For each strip a contraction baseline was acquired to serve as reference.[38]

To administer liposome treatments, 600 µL of PSS buffer was carefully extracted from an organ bath and tranferred to an Eppendorf tube. The appropriate volume of liposome preparation (mixed by inversion) was pipetted into the PSS to pre-dilute the liposomes. The total volume of pre-diluted liposomes (600 µL PSS + liposomes) was then carefully reinjected back into the appropriate organ bath. Final concentrations of each drug were; NIF 7.7 µM, SAL 9.25 µM, ROL 19.4 µM and DOF 3.0 µM. Doses were based on prior investigations of the non-encapsulated drug (*in vitro* contraction assays using human myometrium). Where treated tissue was not washed, tissue strips remained in the presence of the liposomes for the duration of the assay. Where washout studies were performed, organ baths were twice drained of buffer and refilled with 37°C PSS. Human tissue strips were washed after 1 h 25 min whereas mouse tissue strips were washed after 15 min.

Tension generated by tissue strips is indicated in the results and representative contraction traces. The effect of treatments is interpreted relative to the pre-treatment contraction baseline, which consisted of 3 or more contractions of consistent frequency and amplitude.

In vivo biodistribution study

Timed-mated CD1 Swiss pregnant mice were injected with drug-free, DiI-labelled preparations of either non-targeted or OTR-targeted liposomes on fetal gestation days 17 and 18 at 4:00pm. Mice that labored overnight were euthanized on the morning of day 19 (9:00 – 11:00am) by CO_2 asphyxiation. Maternal internal organs of interest (heart, brain, liver, lung, kidney, uterus and mammary tissue) were harvested and transferred to a Petri dish along with a sacrificed neonate. The Petri dish was loaded into an In Vivo Imaging

System (IVIS-100) (Xenogen, CA, USA) and a light image captured. Tissues were then imaged under conditions appropriate for the detection of DiI (Excitation: 554 nm; Emission: 583 nm; Filter: DsRed; Exposure: 4 sec; Field of view: 10; Binning: 4). Organs were imaged 17 – 19 h after the second injection, following labor. Background signal was subtracted from the detected signal to produce the final fluorescence image. Fluorescence signal is reported as radiance (p/sec/cm^2/sr). The radiance range was kept constant across all images (min = 2.0×10^8 : max = 1.8×10^9).

Preterm birth study

Time-mated pregnant CD1 Swiss mice were administered 0.7 µg/g LPS from E. coli (0111:B4) (Sigma-Aldrich) via intraperitoneal (IP) injection at 12:00pm on gestation day 15 (GA15)(one-time injection). LPS dose had been previously optimized to result in PTB rates of 50 – 70%. Total IP injection volume was 150 µL in saline.

At 4:00pm on GA15, mice began receiving daily intravenous (IV) injections of IND free-drug or liposomal preparations according to assigned treatment groups. Treatment groups are indicated in Table 1. Total IV injection volume was 150 µL. Mice were monitored for onset of labor every 6 h. Treatments were repeated daily at 4:00pm until all mice labored. Term gestation was 19 – 22 days. Mice that labored within 48 h of receiving LPS (GA17) were deemed to have labored preterm.

Statistical analyses

For contraction traces, LabChart software was used to determine the area under the curve (AUC) (g tension × sec) for the 30 min prior to treatment (pre-treatment) and 30 min after treatment (post-treatment) (ADInstruments, NZ). AUC before and after treatment was compared by two-tailed paired t-test (GraphPad Prism).

For DOF studies, contraction plateau duration (sec) was determined for four contractions pre- and post-treatment using LabChart software (ADInstruments, NZ). Plateau duration was determined as the time between the point of highest amplitude and point where contraction force declined sharply. Contraction duration data were obtained for 3 individual tissues (n=3 women). Pre- and post-treatment measurements (n=12 each) were compared by two-tailed unpaired t-test.

Average radiance (p/sec/cm^2/sr) was determined for each organ of interest using Living Image software (v2.5). Where fluorescence was detected, regions of interest (ROIs) were applied automatically (contour). Where detection was low or absent, ROIs were specified manually (circles or squares) to tightly encompass the tissue being analyzed. Data were tested for normality by the Shapiro-Wilk normality test (GraphPad Prism). Average radiance for each organ/tissue was compared between treatment groups (n=4 animals per group) by 1-way ANOVA with multiple comparisons (Holm-Sidak) (GraphPad Prism).

For the preterm labor studies, rate of PTB was compared between treatment groups by Chi-squared analysis. Time (h) between LPS injection and labor was calculated. Data were transformed ($Y=Y^2$) to obtain normal distribution (D'Agostino & Pearson normality test) and analyzed by 1-way ANOVA with multiple comparisons (Tukey). Data recorded for number of pups for term deliveries was normally distributed (Shapiro-Wilk normality test) and analyzed by 1-way ANOVA with multiple comparisons (Tukey). Preterm deliveries did not yield any viable pups.

Consumables and reagents

Mice were supplied by the University of Newcastle Animal Support Unit (ASU). Nifedipine (cat#1075), salbutamol hemisulfate (cat#0634), rolipram (cat#0905) and dofetilide

(cat#3757) were purchased from Tocris (Bristol, UK). Indomethacin (cat#L2630) was purchased from Sigma-Aldrich Pty. Ltd (Sydney, Australia). Anti-OTR antibody (ab115664) was purchased from Abcam (Cambridge, MA, USA). Other miscellaneous reagents were purchased from Sigma-Aldrich Pty. Ltd and ThermoFisher Scientific Inc. (Watham, MA, USA).

Results

Characteristics of the liposomal delivery system

OTR-targeted PEGylated immunoliposomes had a mean particle size of 197 ± 6.8 nm with a polydispersity index of 0.243 ± 0.043 (Mean ± S.D.; n=3). The size and polydispersity of the control liposome formulations were similar. Encapsulation of therapeutic agents into the liposomes did not significantly affect the size or polydispersity index. Mean antibody coupling ratio for the OTR-targeted liposomes was 1.86 ± 0.17 µg of antibody per µmol of phospholipid. With a starting antibody concentration of 25 µg and a phospholipid concentration of 2.03×10^{-5} mol this equates to a conjugation efficiency of >99%. The liposomes have a neutral net charge and a drug loading efficiency of >95%, which equates to ~4 mg/mL of drug encapsulated per mL of liposome suspension composed of 16 mg DSPC and 4 mg cholesterol (molar ratio 2:1). *In vitro* dialysis studies have demonstrated highly stable vesicles upon dilution in an aqueous phase (PBS pH 7.4) and in serum (50% FCS) at 37^{o}C (data not included).

Human myometrial contractility

Contraction bioassays were performed to assess whether targeted liposomes were capable of delivering encapsulated therapeutic agents to modulate spontaneous human uterine contractions *in vitro*. Treating the uterine strips with OTR-targeted liposomes that contained

no therapeutic agent (n=3) (Figure 2Ai) had no effect on myometrial contractility in that AUC was not affected (p=0.08; pre-treatment = 1790.0 ± 19.5; post-treatment = 1704.0 ± 38.8 g.sec) (Figure 2Aii). For each therapeutic agent examined *in vitro*, we prepared non-targeted (IgG-coated) and OTR-targeted (anti-OTR-coated) liposomes (at 4 mg/mL). Administering 7.7 μM NIF to human uterine strips via non-targeted NIF-loaded liposomes (Figure 2Bi) (n=5) had no effect on contractility in that AUC the curve was not affected (p=0.4136; pre-treatment = 1701.4 ± 55.9; post-treatment = 1643.8 ± 19.6 g.sec) (Figure 2Bii). Administering 7.7 μM NIF via OTR-targeted NIF-loaded liposomes (Figure 2Biii) (n=4) resulted in abolition of myometrial contractions and a significant reduction in AUC (p=0.0277; pre-treatment = 1767.8 ± 15.5; post-treatment = 1137.5 ± 24.8 g.sec) (Figure 2Biv).

Administering 9.25 μM SAL to spontaneously contracting human uterine strips via non-targeted SAL-loaded liposomes (Figure 2Ci) (n=3) had no effect on contractility with AUC being unaffected by the treatment (p=0.2022; pre-treatment = 1775.3 ± 24.8; post-treatment = 1749.3 ± 16.4 g.sec) (Figure 2Cii). Administering 9.25 μM SAL via OTR-targeted SAL-loaded liposomes (Figure 2Ciii) (n=3) resulted in complete abolition of contractions and significant reduction in AUC (p=0.0293; pre-treatment = 1749.7 ± 27.3; post-treatment = 1292.0 ± 77.1 g.sec) (Figure 2Civ). Similar results were observed when ROL was encapsulated in non-targeted and OTR-targeted liposomes (Figure 4).

To demonstrate that our OTR-targeted liposomes are capable of functioning as a drug delivery system for different obstetric applications, such as treating PPH, liposomes were prepared that contained the hERG channel blocker, DOF. When administered to human myometrial tissue, DOF increases the contraction duration and reduces contraction frequency by delaying repolarization of the myocyte membrane.[36] Administering 3.0 μM DOF to spontaneously contracting tissue strips (n=3) via non-targeted DOF-loaded liposomes (Figure 3Ai) had no significant effect on contraction plateau duration (p=0.083;

pre-treatment = 27.0 ± 0.8; post-treatment = 39.0 ± 3.7 sec) (Figure 3Bi). When administered via OTR-targeted DOF-loaded liposomes (Figure 3Aii), 3.0 μM DOF significantly increased contraction plateau duration (p=0.0001; pre-treatment = 66.4.0 ± 9.8; post-treatment = 162.4 ± 35.4 sec) (Figure 3Bii). Increased contraction plateau duration is consistent with our previous report of DOF action on human myometrium.[36] These results demonstrate that a single delivery system, OTR-targeted liposomes, can be utilized to deliver either contraction blocking or contraction promoting therapeutics to uterine myocytes.

The effect of non-targeted and OTR-targeted DOF-loaded liposomes on the AUC was analyzed. Neither treatment significantly affected AUC (data not shown).

Effect of targeted liposomes is reversible

To demonstrate that the effects on contractility were due to pharmacological actions of the drugs and not the result of toxic effects of OTR-targeted liposomes, washout experiments were performed. ROL is a reversible inhibitor of phosphodiesterase (PDE) IV that induces myometrial relaxation.[39,40] Administering 19.4 μM ROL to contracting strips via non-targeted ROL-loaded liposomes (Figure 4Ai) (n=3) had no effect. When 19.4 μM ROL was administered via OTR-targeted ROL-loaded liposomes (Figure 4Aii) (n=3) contractions were abolished. Analyses of contraction data indicated no reduction in AUC following treatment with non-targeted ROL-loaded liposomes (p=0.061; pre-treatment = 1657.0 ± 21.0; post-treatment = 1611.7 ± 14.9 g.sec) (Figure 4Bi), whereas AUC was significantly reduced following treatment with 19.4 μM ROL administered via OTR-targeted ROL-loaded liposomes (p=0.0023; pre-treatment = 1648.1 ± 14.3; post-treatment = 1155.7 ± 36.3 g.sec) (Figure 4Bii). Once contractions had been inhibited for 1 h 25 min, tissue strips were washed twice in PSS and monitored. Spontaneous, rhythmic contractions resumed in myometrial strips previously treated with OTR-targeted ROL-loaded liposomes (Figure 4Aii), indicating that the tissue remained viable.

Mouse myometrial contractility

Prior to commencing mouse *in vivo* studies, we confirmed that liposomes were effective in delivering therapeutic agents to mouse uterine tissue *in vitro*. Results observed in the mouse were consistent with human myometrial contractility studies. OTR-targeted, drug-free liposomes (a control preparation) had no effect on mouse uterine contractions (Figure 5A) (n=3). Administering 9.25 μM SAL via non-targeted (IgG-coated control) SAL-loaded liposomes had no effect on contractility (Figure 5Bi) (n=3), whereas the same SAL dose administered via OTR-targeted SAL-loaded liposomes abolished mouse myometrial contractions *in vitro* (Figure 5Bii) (n=3). Spontaneous contractions resumed following washing of tissue strips, demonstrating that the mouse uterine tissue remained viable following administration of the liposomes. Similar results were obtained for liposomes loaded with NIF (data not shown). These results demonstrated that OTR-targeted liposomes were effective in modulating mouse myometrial contractility.

Liposome biodistribution

We examined the biodistribution of DiI-labelled non-targeted (naked) and OTR-targeted liposomes that occurred *in vivo*. Pregnant mice were injected with liposomes approaching term (GA17 and 18) then scarified shortly after labor (GA19) (17 – 19 h after injection). Whole organs (liver, brain, heart, kidney, lung, mammary tissue and uterus) were placed on Petri dishes, along with a euthanized neonate, and imaged. The arrangement of tissues (Figure 6A) was kept consistent when imaging tissues from different mice. DiI does not readily exchange out of liposomes into cell membranes or other lipid-containing structures, and therefore is an appropriate marker to assess the biodistribution of liposomes.

Fluorescence detection (p/sec/cm^2/sr) of non-targeted liposomes injected into pregnant mice consistently revealed liposome accumulation in the liver, which is the site of

liposome clearance from the blood stream and metabolism.[41] Accumulation of non-targeted liposomes was not detected in the brain, heart, kidney, lung, mammary tissue or uterus nor in the neonates (n=4 each) (Figure 6Bi). Organs isolated from mice injected with OTR-targeted liposomes showed accumulation of the OTR-targeted liposomes in the uterus and the mammary glands. As expected, there were also high levels of liposome localization in the liver. OTR-targeted liposome accumulation was not detected in the brain, heart, kidney or lung, nor in the neonates (n=4 each) (Figure 6Bii).

DiI fluorescence was quantified for each organ (Table 2). OTR targeting of liposomes resulted in significantly increased localization to the uterus compared to non-targeted liposomes (p<0.0001; non-targeted = $5.04 \times 10^7 \pm 7.5 \times 10^6$; OTR-targeted = $3.57 \times 10^8 \pm 3.05 \times 10^7$ p/sec/cm^2/sr) (Figure 6C). On average this equalled a 7-fold increase in uterine localization. Furthermore, the level of OTR-targeted liposome localization in the uterus was significantly greater than that of brain (p=0.0002), lung (p=0.0003), kidney (p=0.0005), heart (p<0.0001) and neonate (p<0.0001) (Figure 6C). For both non-targeted and OTR-targeted liposomes, accumulation in the liver was significantly greater than all other organs examined (p<0.0001), however there was no difference in liposome accumulation in the liver between non-targeted and OTR-targeted liposomes (p>0.9999; non-targeted = $7.90 \times 10^8 \pm 9.15 \times 10^7$; OTR-targeted = $7.37 \times 10^8 \pm 9.05 \times 10^7$) (Figure 6C).

Preventing preterm birth

We used an LPS model of PTB to access whether targeted liposomes could be used to administer IND for the prevention of LPS-induced PTB in mice. Non-targeted and OTR-targeted liposomes loaded with 5.5 mg/mL IND were compared against IND administered as free-drug (1.0 or 2.0 mg/kg/day). Observed PTB rates are indicated in Table 3. Chi-squared analyses were performed.

PTB rates in control mice (Group 1) and the LPS control group (Group 2) were 0 (n=12) and 67% (n=18), respectively. At 2.0 mg/kg/day, IND administered as free-drug significantly reduced rates of PTB from 67% down to 31% (Group 2 (n=18) vs Group 4 (n=13); p=0.0484) (Figure 7A). PTB rate for OTR-targeted, drug-free control liposomes (Group 5) was 56% (n=16), and was not different to PTB rate observed for LPS control animals (Group 2) (p=0.532). IND administered at 2.0 mg/kg/day via non-targeted liposomes (Group 6) (n=12) had no effect as the observed PTB rate of 58% was not significantly different to PTB rates for LPS control animals (Group 2) (p=0.643) or animals treated with OTR-targeted, drug-free liposomes (Group 5) (p=0.91) (Figure 7A).

IND administered at 2.0 mg/kg/day via OTR-targeted liposomes (Group 7) (n=11) resulted in a PTB rate of 18%, which was a significant reduction compared to the PTB rate of 67% for the LPS control animals (Group 2) (p=0.0112) (Figure 7A). Furthermore, PTB rate for 2.0 mg/kg/day IND administered via OTR-targeted liposomes was significantly reduced compared to the same dose administered by non-targeted liposomes (Group 6) (p=0.048). No significant difference was observed between 2.0 mg/kg/day IND admnistrered as free-drug compared to when administered via OTR-targeted liposomes (Group 4 vs Group7; p=0.4780) (Figure 7A).

The time between LPS injection and labor was calculated for each animal (average ± SEM shown in Table 3). Analysis of the normalised data showed that IP administration of LPS (0.7 µg/g) significantly advanced the time of labor, compared to control animals (Group 1 vs Group 2; p=0.0017)(Figure 7B). IND administered as free-drug at 1.0 and 2.0 mg/kg/day dose-dependently increased the average time between LPS injection and labor (77.5 ± 14.1 and 86.6 ± 12.7 h, respectively) compared to the LPS control (50.8 ± 8.9 h), however neither dose reached statistical significance (Group 2 vs Group 3; p=0.53) (Group 2 vs Group 4; p=0.08) (Figure 7B). OTR-targeted, drug-free liposomes had no effect on time between LPS injection and labor, compared to LPS control animals (Group 2 vs Group 5; p=0.92). 2.0

mg/kg/day IND administered via non-targeted liposomes had no significant effect on the time between LPS injection and labor (Group 2 vs Group 6; p=0.99), however, when administered via OTR-targeted liposomes, time between LPS injection and labor was significantly increased (Group 2 vs Group 7; p=0.0048). The time between LPS injection and labor was significantly different between 2.0 mg/kg/day IND delivered via non-targeted liposomes compared to OTR-targeted liposomes (Group 6 vs Group 7; p=0.0438).

Number of live pups was recorded for term deliveries (no viable pups arose from preterm deliveries). Data were normally distributed (Shapiro-Wilk normality test) and analyzed by 1-way ANOVA with multiple comparisons (Tukey). There was no significant difference in the number of live pups from term deliveries in the different groups (Figure 7C).

Comment

Principal Findings

OTRs are expressed at low levels on various tissues toward the end of pregnancy, including the brain and mammary tissue.[42] Expression in the pregnant uterus however is high approaching term,[30,31] indicating that the OTR is an excellent candidate for the development of a targeted drug delivery system for the uterus. This study represents an initial analysis of OTR-targeted liposomes as a drug delivery system, and demonstrates that:

(i) conjugation of the OTR antibody to the surface of liposomes confers the ability for NIF-, SAL- and ROL-loaded liposomes to significantly reduce human myometrial contractions *in vitro*, as confirmed by AUC analyses,

(ii) enhancement of myometrial contractility can be achieved through encapsulation of uterotonic agents, as confirmed by use of OTR-targeted DOF-loaded liposomes to significantly increase contraction plateau duration,

(iii) non-targeted liposomes loaded with these same therapeutic agents do not affect myometrial contractions *in vitro*, as confirmed by AUC and contraction plateau duration analyses,

(iv) the effects are reversible (depending on the therapeutic), as confirmed by the spontaneous resumption of contractions in both human and mouse myometrial tissue *in vitro*,

(v) the OTR-targeted liposomes themselves have no apparent effect on myometrial contractions, as confirmed by AUC analyses for myometrial contractions in vitro, and lack of effect on PTB rates in mice or time between LPS injection and labor,

(vi) *in vivo,* OTR-targeted liposomes localize to the uterus and breast of pregnant mice whereas non-targeted liposomes do not. Uterine localization was increased 7-fold by OTR targeting, as confirmed by quantitation of average radiance for key organs of interest,

(vii) no evidence of transplacental passage of the liposomes to the fetus was observed, as determined quantitative evaluation of DiI fluorescence in neonates,

(viii) OTR-targeted liposomes loaded with IND are effective in reducing rates of LPS-induced PTB in mice whereas non-targeted IND-loaded liposomes have no effect.

Clinical Significance

Many current tocolytics have been associated with adverse effects on the mother (β-sympathomimetics)[6,43,44] and on the fetus (NIF, IND)[16-18,45,46] or have no evident effect on prolongation of pregnancy (atosiban).[14,15] NIF is capable of providing some clinical benefit, with a systematic review and meta-analysis indicating a significant reduction in the risk of delivery within 7 days of initiation of NIF treatment.[3] However, the high doses required to achieve relaxation of the myometrium increases the risk of adverse systemic effects.[45-47] Indomethacin has been explored as a tocolytic agent for preterm labor,[48-50] however,

systematic review indicates that indomethacin is associated with an increased risk for severe intraventricular hemorrhage, necrotizing enterocolitis, and periventricular leukomalacia[51]. A recent study by Refuerzo et al. (2015) demonstrated that encapsulation of IND inside non-targeted liposomes can reduce IND levels in the fetus 7.6-fold, suggesting the potential for reduced fetal side effects.[52]

Here we have demonstrated in mice that IND encapsulated inside OTR-targeted liposomes was effective in reducing rates of LPS-induced preterm birth, whereas non-targeted IND-loaded liposomes were not. These results, in conjunction with our biodistribution studies, suggest that OTR-targeting confers upon liposomes the ability to target therapeutics to the uterus. The clinical implications are that OTR-targeted liposomes may enable existing tocolytics, such as NIF and IND, to be administered with improved efficacy and improved safety.

The restricted biodistribution of OTR-targeted liposomes raises the possibility of introducing into clinical practice therapeutic agents that are known to be highly effective tocolytics, yet are known to have adverse off-target effects. One such group of candidates are the PDE4 inhibitors, such as ROL, which have been demonstrated to be highly effective in controlling inflammation-driven preterm delivery in mice.[53] Mounting evidence indicates that PTB in humans is also an inflammation driven event, and evidence that ROL is highly effective in abolishing spontaneous contractions in human myometrium[33] suggests that PDE4 inhibitors may be an excellent cargo for OTR-targeted liposomes in the setting of preterm labor.

Clinical implications also include the prospect of encapsulating uterotonic agents, and here we demonstrate that possibility through the use of DOF. DOF is not a traditional uterotonic agent, but when administered via OTR-targeted liposomes DOF significantly increased the duration of human myometrial contractions *in vitro*, consistent with previous findings.[36] Targeted delivery of uterotonics may be useful to promote contractions, including

during failure of labor to progress, expulsion of the placenta after labor, expulsion of retained products after miscarriage, or to control PPH. PPH is a leading cause of maternal mortality world-wide and is linked with major morbidities such as peripartum hysterectomy and massive transfusion.[54-56] First line therapy for PPH is uterine massage and oxytocin administration, however rates of atonic PPH after oxytocin use are increasing in many developed countries.[57,58] When refractory uterine atony occurs, second line therapy may include administration of uterotonic agents such as methylergonovine and carboprost. Methylergonovine was recently identified as the more effective of the two,[59] however both agents could effectively be encapsulated in OTR-targeted liposomes. Evidence indicates that post-receptor contractile signalling pathways are maintained in oxytocin desensitized primary myocytes *in vitro*,[60] however oxytocin desensitization occurs, at least in part, by down-regulation of OTR protein levels.[61] Uterotonic-loaded liposomes targeted to the oxytocin receptor may therefore be of reduced effectiveness in patients with prolonged exposure to oxytocin.

Future Research

These data provide the first evidence that OTR-targeted liposomes are a drug delivery system that affords flexibility in delivery of different classes of therapeutic agents to human uterine tissue in order to modulate myometrial contractility. Furthermore, these data provide the first evidence that OTR-targeted liposomes can be used to administer therapeutic agents for the prevention of preterm birth in mice.

Further studies are necessary to determine the mechanism of OTR-targeted liposome uptake in myocytes, the quantitative biodistribution of therapeutic agents achieved in the uterus compared to other organs, and the rate of liposome clearance. Additional studies have been planned to determine whether the use of OTR-targeted liposomes to administer therapeutics for prevention of preterm birth is effective in reducing fetal side

effects, such as premature closure of the ductus arteriosus in response to IND exposure in utero.

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Author Contributions

Conceived and designed experiments: JP, RS, SH. Liposome manufacture: SH. Animal studies: JP, MI, JT. Sample collection: JP, MI, TB. Contraction bioassays: JP, MI, TB. Data Analysis: JP, MI. Provided reagents and materials: RS, SH, JP. Manuscript Writing: JP, SH, MI, JT, TB, RS.

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Tables

Table 1. Treatment groups for in preterm labor study

Group (n)	One-time IP Injection (12:00pm on GA15, 150 µL)	Daily IV injections (4:00pm, GA15 onwards, 150 µL)
1	saline	saline
2	0.7 µg/g LPS	50% DMSO
3	0.7 µg/g LPS	1.0 mg/kg/day IND in 50% DMSO
4	0.7 µg/g LPS	2.0 mg/kg/day IND in 50% DMSO
5	0.7 µg/g LPS	OTR-targeted, drug-free liposomes in saline
6	0.7 µg/g LPS	2.0 mg/kg/day IND via non-targeted liposomes in saline
7	0.7 µg/g LPS	2.0 mg/kg/day IND via OTR-targeted liposomes in saline

LPS, lipopolysaccharide; *OTR*, oxytocin receptor; *IP*, intraperitoneal; *IV*, intravenous; *GA15*, pregnancy day 15

Table 2. Average radiance of DiI-labelled liposomes detected in organs and neonates

Organ / tissue	Average radiance (p/sec/cm2/sr) (mean ± SEM)	
	Non-targeted liposomes (n=4 animals)	OTR-targeted liposomes (n=4 animals)
Liver	$9.73 \times 10^8 \pm 9.1 \times 10^7$	$7.37 \times 10^8 \pm 9.05 \times 10^7$
Uterus	$5.04 \times 10^7 \pm 7.5 \times 10^6$	$3.57 \times 10^8 \pm 3.05 \times 10^7$
Mammary tissue	$7.29 \times 10^7 \pm 6.05 \times 10^6$	$1.78 \times 10^8 \pm 6.47 \times 10^7$
Brain	$3.97 \times 10^7 \pm 2.51 \times 10^6$	$7.03 \times 10^7 \pm 4.51 \times 10^6$
Lung	$4.65 \times 10^7 \pm 4.82 \times 10^6$	$7.97 \times 10^7 \pm 2.94 \times 10^6$
Kidney	$3.46 \times 10^7 \pm 1.78 \times 10^6$	$8.79 \times 10^7 \pm 9.51 \times 10^6$
Heart	$2.79 \times 10^7 \pm 1.65 \times 10^6$	$5.52 \times 10^7 \pm 1.65 \times 10^6$
Neonate	$-4.83 \times 10^7 \pm 1.30 \times 10^7$	$-1.03 \times 10^7 \pm 1.46 \times 10^7$

OTR, oxytocin receptor

Table 3. Rates of LPS-induced preterm birth and time between LPS injection and labor

Group No.	Treatment Group	n	PTB rate (%)	Time between LPS injection and observed labor (h) (mean ± SEM)
1	Control (no LPS, no liposomes)	12	0/12 (0)	109.7 ± 4.1
2	LPS control (+ 50% DMSO)	18	12/18 (67)	50.8 ± 8.9
3	LPS + 1.0 mg/kg IND (50% DMSO)	10	4/10 (40)	77.5 ± 14.1
4	LPS + 2.0 mg/kg IND (50% DMSO)	13	4/13 (31)	86.6 ± 12.7
5	LPS + OTR-targeted, drug-free liposomes	16	9/16 (56)	65.0 ± 9.9
6	LPS + Non-targeted 2.0 mg/kg IND liposomes	12	7/12 (58)	55.6 ± 12.4
7	LPS + OTR-targeted 2.0 mg/kg IND liposomes	11	2/11 (18)	101.3 ± 12.4

OTR, oxytocin receptor; *PTB*, preterm birth; *LPS*, lipopolysaccharide; *IND*, indomethacin; DMSO, dimethyl sulfoxide

Figure Legends

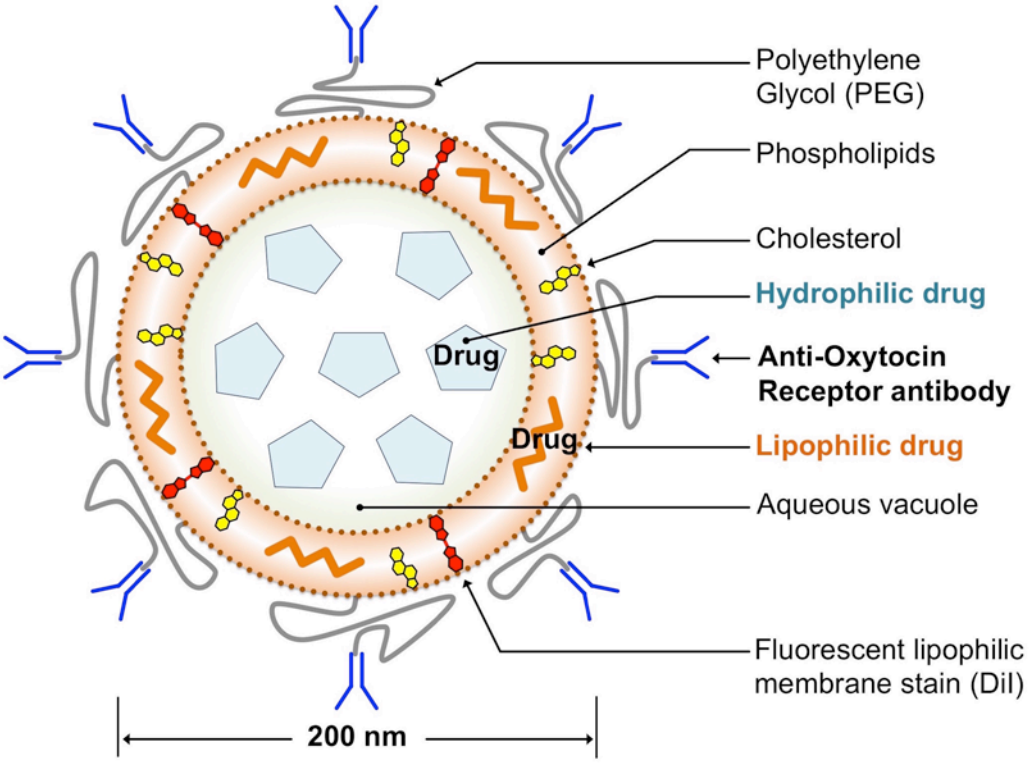

Figure 1. Schematic of OTR-targeted liposome structure

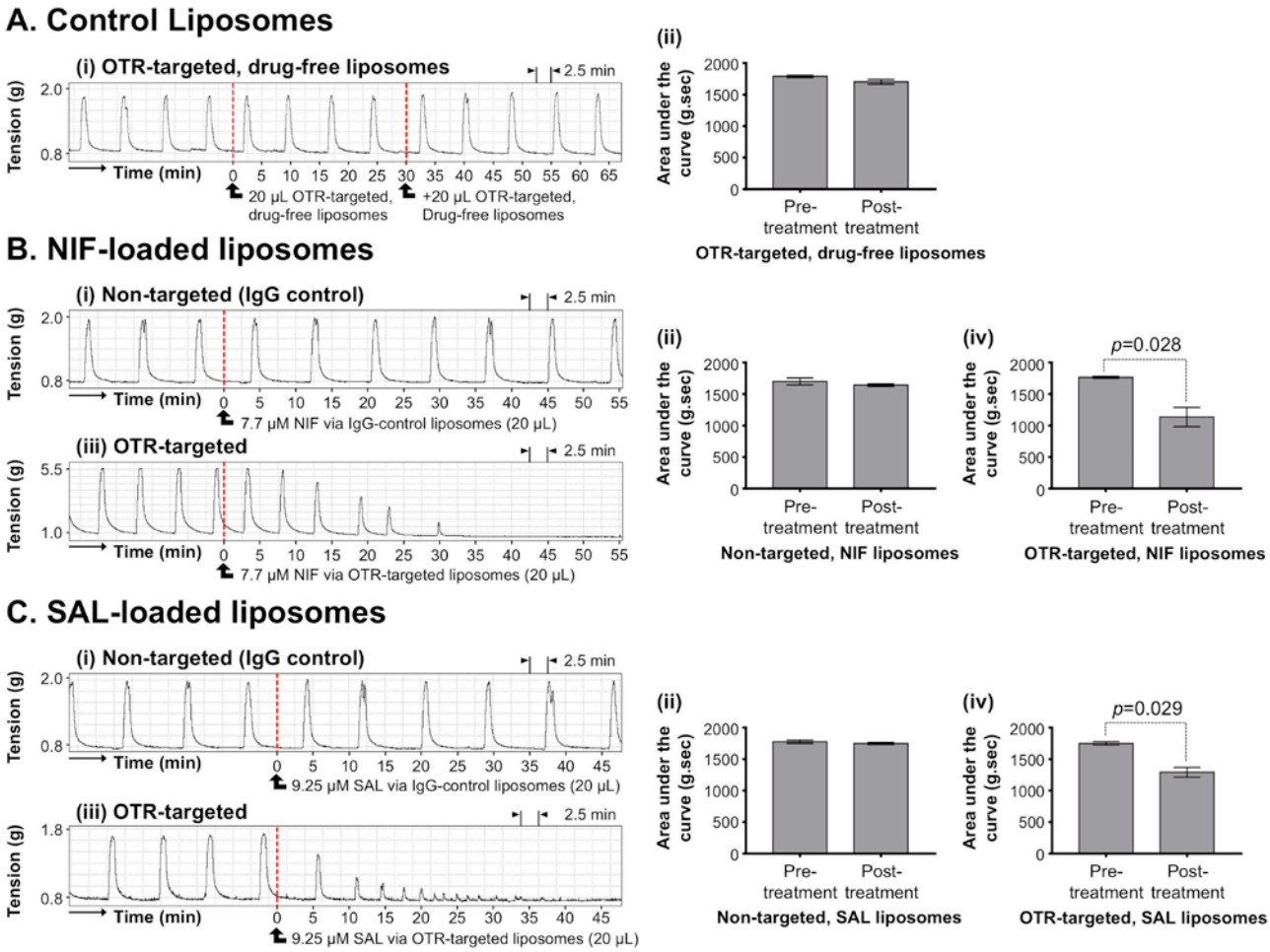

Figure 2. Use of targeted liposomes to inhibit human uterine contractility *in vitro*. Data are contraction traces for strips of human myometrial tissue and corresponding AUC analyses. (A) Effect of OTR-targeted, drug-free control liposomes on myometrial contractions *in vitro* (n=3). (B) Effect of non-targeted (n=5) and OTR-targeted (n=4) NIF-loaded liposomes on myometrial contractions *in vitro*. (C) Effect of non-targeted (n=3) and OTR-targeted (n=3) SAL-loaded liposomes on myometrial contractions *in vitro*. Average AUC analyses covers 30 min immediately prior to and 30 min after treatment with NIF- or SAL-loaded liposomes (pre- and post-treatment, respectively). *AUC analyses are paired t-tests*.

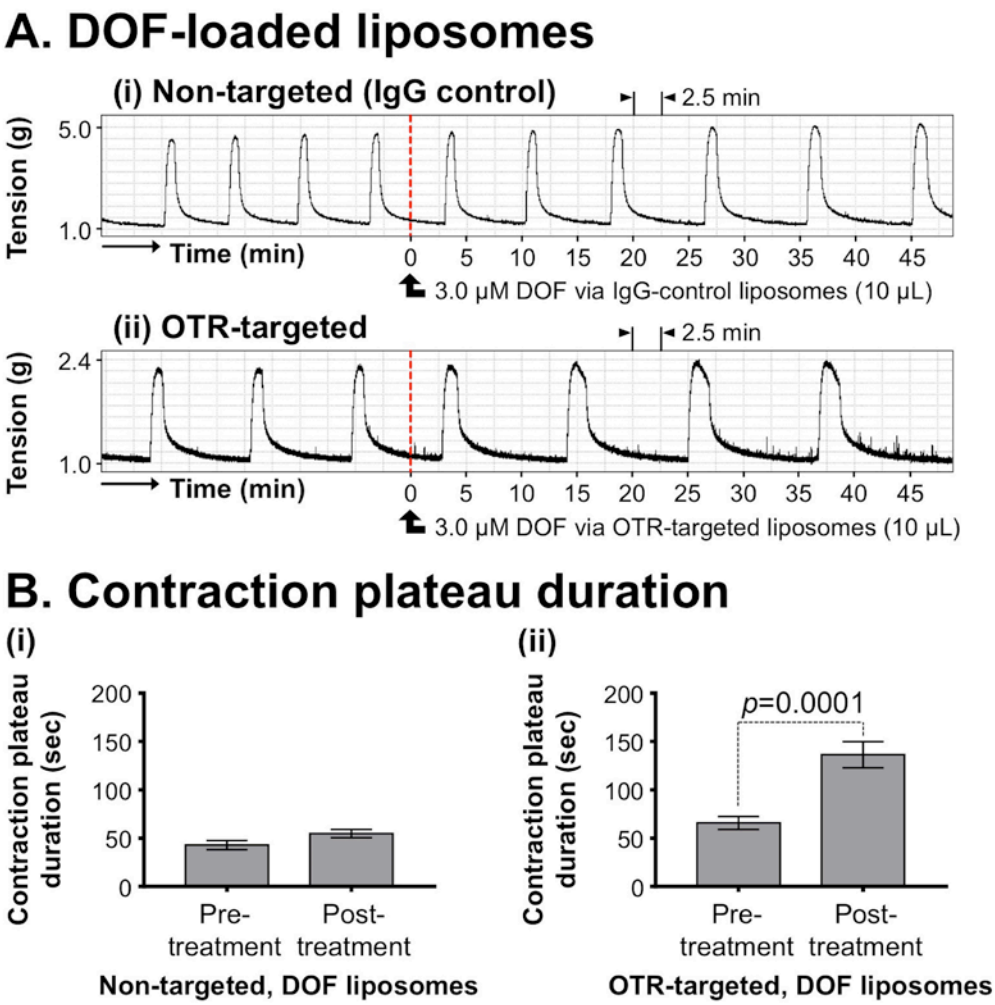

Figure 3. Use of targeted liposomes to enhance human myometrial contractions *in vitro*. Data are contraction traces analyses for strips of human myometrial tissue. (A) Effect of 3.0 μM DOF administered via non-targeted (n=3) or OTR-targeted (n=3) DOF-loaded liposomes on contractility *in vitro*. (B) Average contraction plateau duration for 4 contractions immediately prior to and after treatment with non-targeted or OTR-targeted DOF-loaded liposomes (pre- and post-treatment, respectively) (n=3 tissues strips each). *Unpaired t-test (12 pre-treatment plateau durations vs 12 post-treatment plateau durations).*

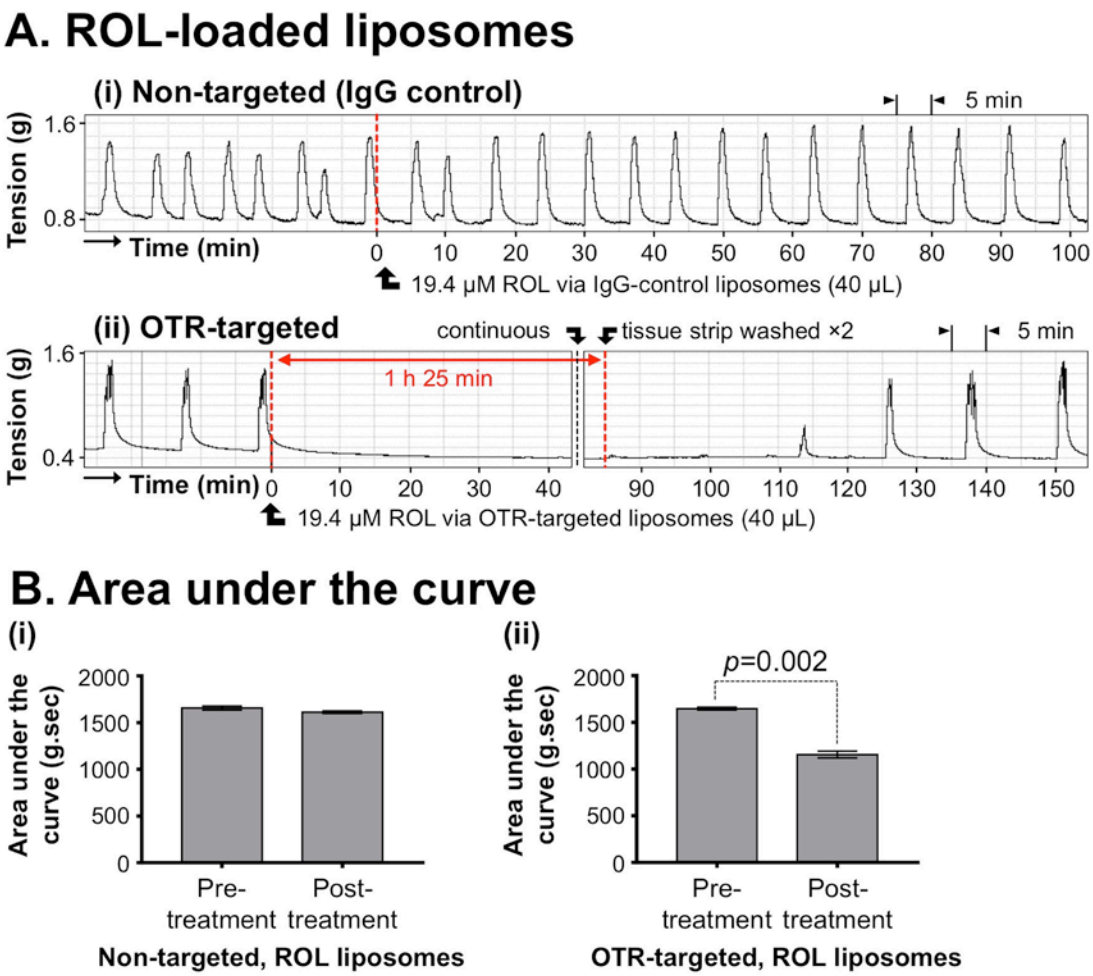

Figure 4. Modulation of uterine contractility by targeted liposomes is reversible. Data are contraction traces for individual strips of human myometrial tissue (A) Effect of non-targeted (n=3) and OTR-targeted ROL-loaded liposomes (n=3) on myometrial contractions *in vitro*. (Aii) demonstrates restoration of contractions after washout. (B) Average AUC for 30 min immediately prior to and 30 min after treatment with ROL-loaded liposomes (pre- and post-treatment, respectively). *AUC analyses were paired t-tests*.

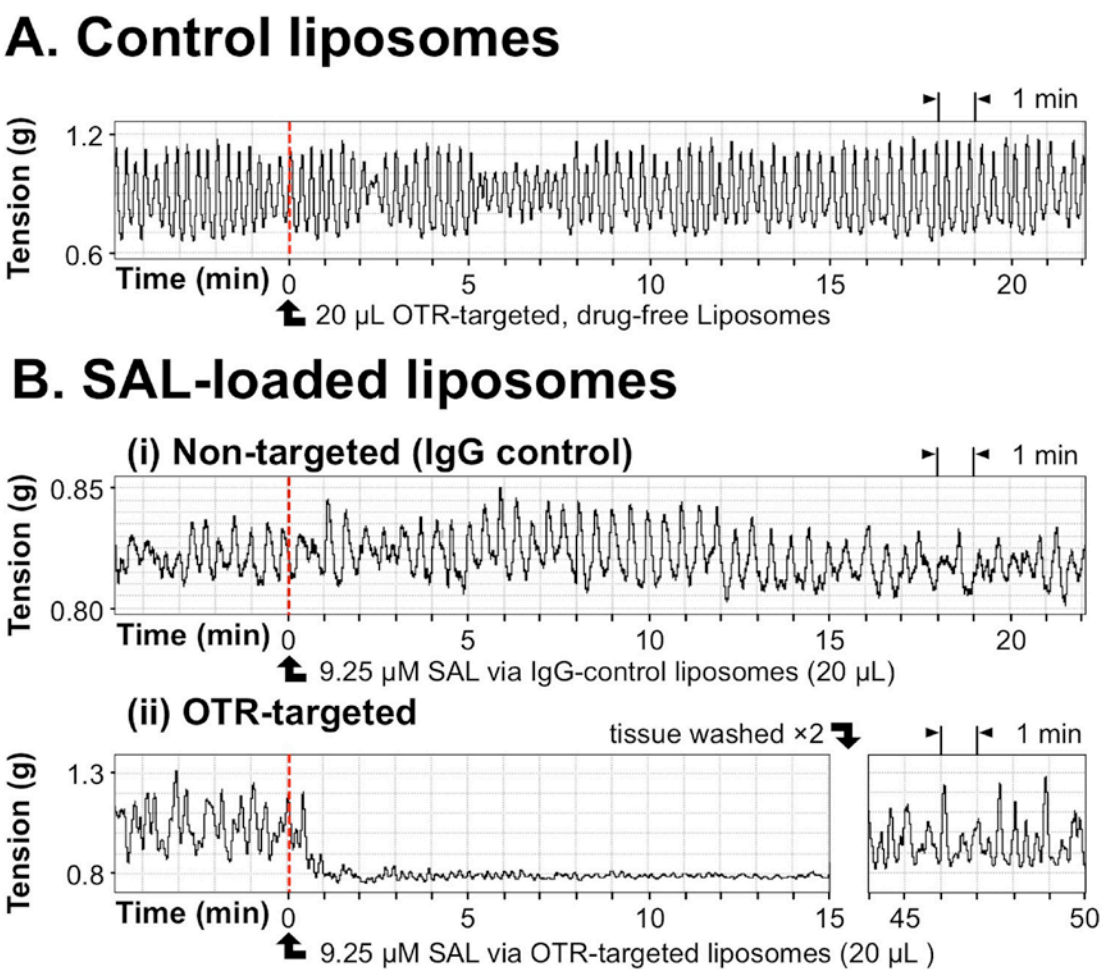

Figure 5. Use of targeted liposomes to modulate mouse uterine contractility *in vitro*. Data are contraction traces for individual strips of mouse uterine tissue and illustrate effect of treatments on contraction amplitude and frequency. (A) Effect of OTR-targeted, drug-free liposomes (n=3). (Bi) Effect of 9.25 µM SAL administered via non-targeted SAL-loaded liposomes (n=3). (Bii) Effect of 9.25 µM SAL administered via OTR-targeted SAL-loaded liposomes (n=3).

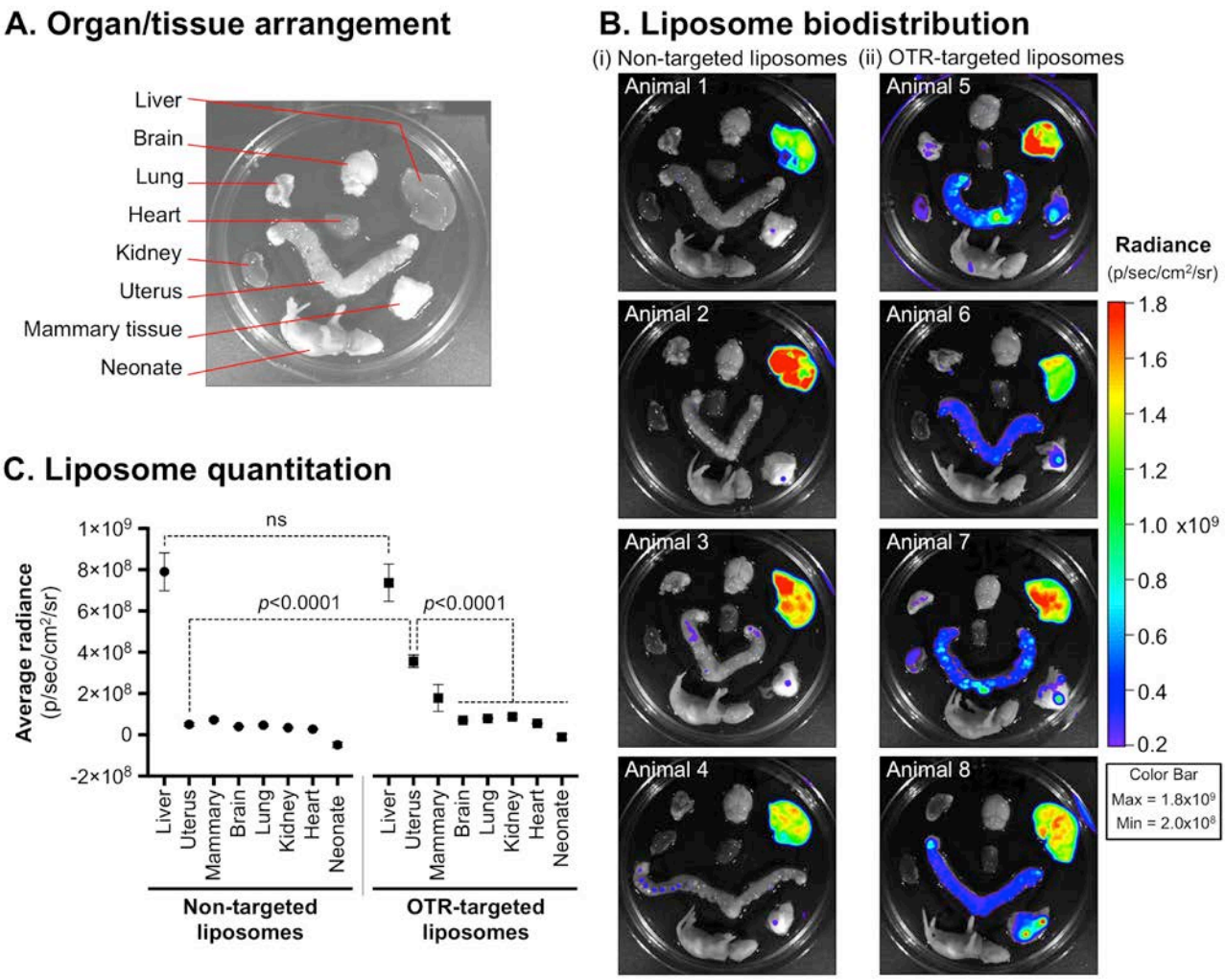

Figure 6. OTR-targeted liposomes accumulate in the uterus *in vivo*. Data are light or fluorescence images captured shortly after labor by an IVIS-100 illustrating liposome biodistribution that occurred *in vivo*. (A) Representative image demonstrating the arrangement of organs and tissues of interest (liver, brain, lung, heart, kidney, uterus, mammary tissue and a neonate). (B) Fluorescent detection of liposome biodistribution that occurred *in vivo*. (Bi) Biodistribution of non-targeted liposomes (n=4). (Bii) Biodistribution of OTR-targeted liposomes (n=4). (C) Quantitation of liposomal detection in different organs. Average radiance (p/sec/cm^2/sr) was determined for each organ and compared across treatment groups (n=4 for each organ per group). *Data were confirmed to be normally distributed (Shapiro-Wilk normality test) then compared by 1-way ANOVA with multiple comparisons (Holm-Sidak). Not all statically significant comparisons are indicated.*

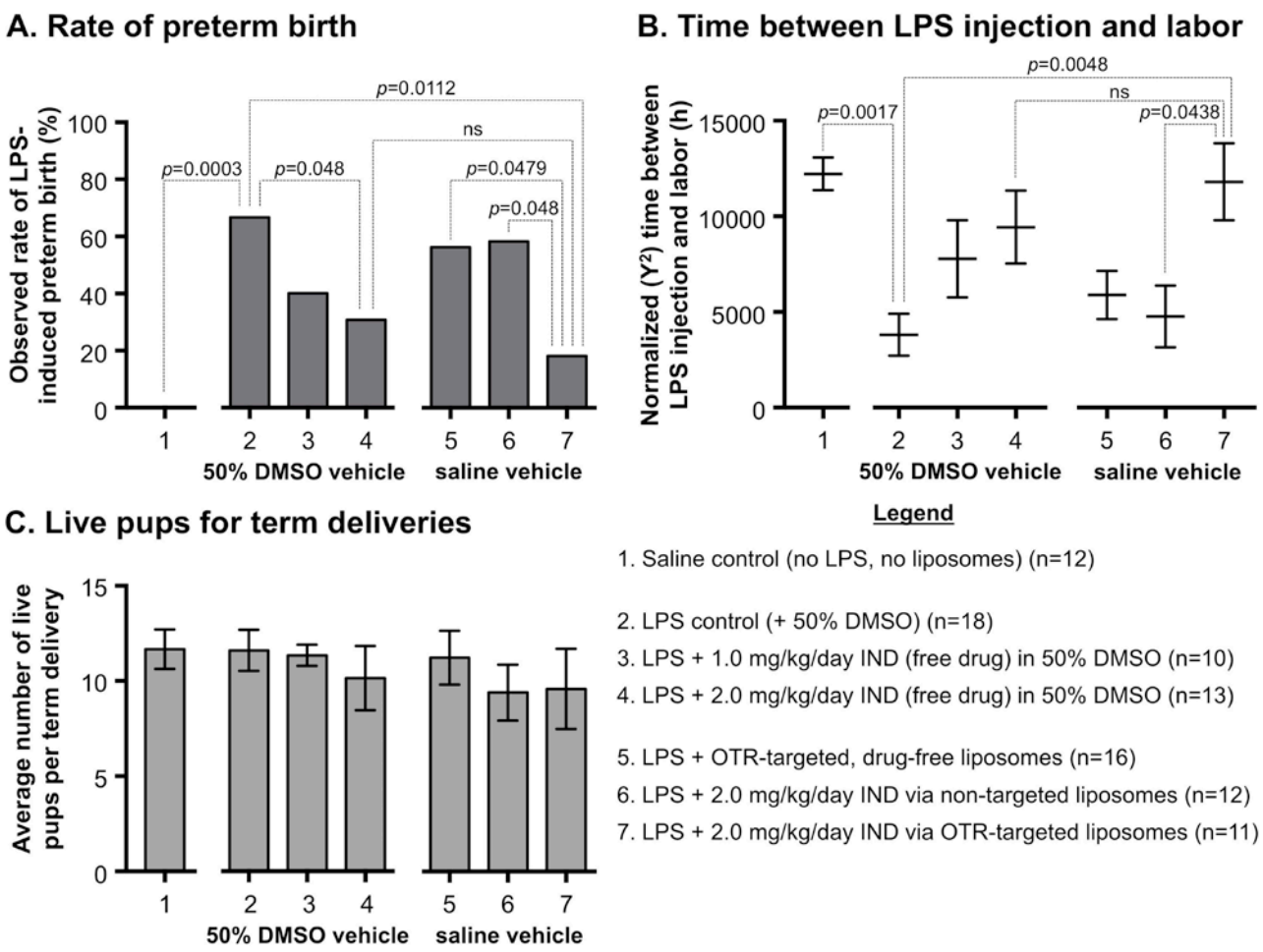

Figure 7. Preventing preterm birth using OTR-targeted indomethacin-loaded liposomes. Efficacy of targeted liposomes was assessed using a LPS mouse model of preterm birth. (A) The effect of IND administered as free drug (1.0 or 2.0 mg/kg/day) or via liposomal preparations (2.0 mg/kg/day) on rates of LPS-induced preterm birth. (B) Time (h) between LPS injection and observed labor. (C) The number of live pups born for term deliveries. No significant differences were recorded in the number of live pups. *Preterm birth rates were analyzed by Chi-square analysis. Data for time (h) between LPS injection and labor were normalized ($Y=Y^2$) (D'Agostino & Pearson normality test) then analyzed by 1-way ANOVA with multiple comparisons (Tukey). Number of live pups was normally distributed and analyzed by 1-way ANOVA with multiple comparisons (Tukey).*