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OBE022, an Oral and Selective Prostaglandin $F_{2\alpha}$ Receptor Antagonist as an Effective and Safe Modality for the Treatment of Preterm Labor[S]

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ABSTRACT

Preterm birth is the major challenge in obstetrics, affecting ~10% of pregnancies. Pan-prostaglandin synthesis inhibitors [nonsteroidal anti-inflammatory drugs (NSAIDs)] prevent preterm labor and prolong pregnancy but raise concerns about fetal renal and cardiovascular safety. We conducted preclinical studies examining the tocolytic effect and fetal safety of the oral prodrug candidate OBE022 [(*S*)-2-amino-3-methyl-butyric acid (*S*)-3-{[(S)-3-(biphenyl-4-sulfonyl)-thiazolidine-2-carbonyl]-amino}-3-(4-fluoro-phenyl)-propyl ester] and its parent OBE002 [(*S*)-3-(biphenyl-4-sulfonyl)-thiazolidine-2-carboxylic acid [(S)-1-(4-fluoro-phenyl)-3-hydroxy-propyl]-amide], both potent and highly selective antagonist of the contractile prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) receptor (FP). Efficacy of OBE022 and OBE002, alone and in combination with other tocolytics, was assessed in human tissues and pregnant animal models for inhibition of uterine contraction and delay of parturition. Selective safety of OBE022 and/or OBE002, compared with NSAID indomethacin, was assessed on renal function, closure of the ductus arteriosus, and inhibition of platelet aggregation. In in vitro studies, OBE002 inhibited spontaneous, oxytocin- and $PGF_{2\alpha}$-induced human myometrial contractions alone and was more effective in combination with atosiban or nifedipine. In in vivo studies, OBE022 and OBE002 reduced spontaneous contractions in near-term pregnant rats. In pregnant mice, OBE022 delayed RU486 [(8S,11R,13S,14S,17S)-11-[4-(dimethylamino)phenyl]-17-hydroxy-13-methyl-17-prop-1-ynyl-1,2,6,7,8,11,12,14,15,16-decahydrocyclopenta[a]phenanthren-3-one] -induced parturition and exerted synergistic effects in combination with nifedipine. OBE022 and/or OBE002 did not show the fetal side effects of ductus arteriosus constriction, impairment of kidney function, or inhibition of platelet aggregation observed with indomethacin. Orally active OBE022 and OBE002 exhibits potent tocolytic effects on human tissues ex vivo and animal models in vivo without causing the adverse fetal side effects seen with indomethacin. Selectively targeting the FP receptor in combination with existing tocolytics may be an effective strategy for preventing or delaying preterm delivery.

Introduction

Preterm birth, defined as delivery before 37 weeks of gestation, is the major cause of perinatal morbidity and mortality worldwide (Slattery and Morrison, 2002). Although the physiologic processes of human labor and parturition are complex and not fully understood, it is recognized that an increase in uterine contractions and shortening and dilatation of the cervix are hallmarks of active labor. Tocolytic agents, drugs targeting physiologic pathways that inhibit uterine contractions, have been used clinically for short-term treatment to allow for completion of a course of corticosteroids for fetal lung

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ABBREVIATIONS: AA, aorta artery; ANOVA, analysis of variance; AS604872, (2S)-3-(4-phenylphenyl)sulfonyl-N-[(R)-phenyl(pyridin-2-yl)methyl]-1,3-thiazolidine-2-carboxamide; AUC, area under the curve; BW245C, 7-[(4S)-3-[(3R)-3-cyclohexyl-3-hydroxypropyl]-2,5-dioxoimidazolidin-4-yl]heptanoic acid; CHO, Chinese hamster ovary; COX, cyclooxygenase; D1, day 1; DA, ductus arteriosus; DMEM-F-12, Dulbecco's modified Eagle's medium F-12 cell culture medium; DMSO, dimethylsulfoxide; EP, prostaglandin E_2 receptor; FF, filtration fraction; FP, prostaglandin $PGF_{2\alpha}$ receptor; FPA, final platelet aggregation; GFR, glomerular filtration rate; HTRF, homogeneous time-resolved fluorescence; IN, inulin; % IPA, percentage inhibition of aggregation; IVH, intraventricular hemorrhage; MMP, matrix metalloproteinase; MPA, maximum platelet aggregation; NP3S, 5% *N*-methylpyrrolidone, 30% polyethylene glycol 400, 25% polyethylene glycol 200, 20% propylene glycol, and 20% saline; NSAID, nonsteroidal anti-inflammatory drug; OBE002, (*S*)-3-(biphenyl-4-sulfonyl)-thiazolidine-2-carboxylic acid [(S)-1-(4-fluoro-phenyl)-3-hydroxy-propyl]-amide; OBE022, (*S*)-2-amino-3-methyl-butyric acid (*S*)-3-{[(S)-3-(biphenyl-4-sulfonyl)-thiazolidine-2-carbonyl]-amino}-3-(4-fluoro-phenyl)-propyl ester, hydrochloride; OT, oxytocin; PAH, para-aminohippurate; PEG400, polyethylene glycol 400; PG, prostaglandin; PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PRP, platelet-rich plasma; RBF, renal blood flow; RPF, renal plasma flow; RU486, [(8S,11R,13S,14S,17S)-11-[4-(dimethylamino)phenyl]-17-hydroxy-13-methyl-17-prop-1-ynyl-1,2,6,7,8,11,12,14,15,16-decahydrocyclopenta[a]phenanthren-3-one]); RVR, renal vascular resistance; THG113.31, H-D-Ile-D-Leu-Gly-D-His-D-Cit-D-Asp-D-Tyr-D-Lys-OH; U44069, (Z)-7-[(1R,2S,3R,4S)-2-[(E,3S)-3-hydroxyoct-1-enyl]-5-oxabicyclo[2.2.1]heptan-3-yl]hept-5-enoic acid; UFR, urinary flow rate.

maturation and to allow transport in utero to a tertiary care facility. However, there is no clear evidence that current tocolytics improve neonatal outcome (Kam and Lamont, 2008). Preterm birth is often associated with inflammation, and this is a major risk for adverse neonatal outcome. Therefore, an ideal tocolytic should have some anti-inflammatory properties.

Prostaglandins, as local mediators of inflammation, play a major role during human pregnancy and parturition (Challis et al., 2002; Slater et al., 2002; Olson, 2005). Levels of prostaglandins in uterine tissues are controlled by a balance between synthesis [via cyclooxygenase 1 (COX_1), COX_2 and specific prostaglandin synthases], and metabolism via prostaglandin dehydrogenase enzymes.

Prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) are the key prostaglandins produced in pregnant myometrium or the adjacent decidua. They play a pivotal role in the initiation and progression of normal and preterm labor by eliciting myometrial contractile activity and cervical maturation (Olson and Ammann, 2007). PGE_2 has complex dual effects upon the uterus. Acting through prostaglandin E_2 receptor 3 (EP_3), PGE_2 mediates contractions (Arulkumaran et al., 2012). PGE_2 exerts a relaxatory effect on the uterus through EP_2 and EP_4 (Kandola et al., 2014). $PGF_{2\alpha}$ contracts the myometrium through activation of prostaglandin $PGF_{2\alpha}$ receptor (FP), which is expressed in term human myometrium (Senior et al., 1993; Leonhardt et al., 2003; Grigsby et al., 2006). $PGF_{2\alpha}$ has been shown to induce upregulation of matrix metalloproteinase (MMP_1), an enzyme that breaks down collagen in cervical fibroblasts, leading to cervical ripening (Yoshida et al., 2002). $PGF_{2\alpha}$ upregulates MMP_2 and MMP_9 and downregulates their naturally occurring tissue inhibitor of metalloproteinases ($TIMP_1$) in human term decidua, thus accelerating the breakdown of collagen and the rupture of membranes (Ulug et al., 2001).

Prostaglandin synthesis inhibitors [nonsteroidal anti-inflammatory drugs (NSAIDs)], such as indomethacin, have been used and are still used as tocolytics in the United States in preterm labor patients before week 32 of pregnancy. Indomethacin was ranked with the highest probability of delaying preterm birth (Haas et al., 2009), but other reports revealed an association between indomethacin or other NSAIDs and vasoconstriction, leading to adverse fetal effects, including oligohydramnios, ductus arteriosus dysfunction, renal function impairment, necrotizing enterocolitis, intraventricular hemorrhage (IVH), and persistent pulmonary hypertension of the newborn (Norton, 1997; Peruzzi et al., 1999; Loudon et al., 2003; Hammers et al., 2015). Indomethacin inhibits both COX_1 and COX_2 and, therefore, the formation of all prostaglandins including the primary fetal prostaglandin, PGE_2. Taken together, these findings suggest that selectively blocking FP activation may be beneficial for the control of preterm labor without affecting the action of other prostaglandins (PG) which may be crucial for fetal development.

There are few reports of tocolytic agents selectively targeting the FP receptor for the inhibition of preterm labor. THG113.31 ([H-D-Ile-D-Leu-Gly-D-His-D-Cit-D-Asp-D-Tyr-D-Lys-OH]; PDC31), a peptide derived from the sequence of the second extracellular loop of FP, was reported to delay preterm birth in mice and sheep (Peri et al., 2002; Hirst et al., 2005). However, the lack of effect of THG113.31 on contractility induced by $PGF_{2\alpha}$ in pregnant human myometrium strips raised questions about its mechanism of action (Friel et al., 2005). Later, peptidomimetic compounds derived from THG113.31 were characterized as allosteric modulators of FP with biased signaling properties in cell lines and some activity in a mouse preterm parturition model (Goupil et al., 2010; Bourguet et al., 2011). THG113.31 showed some effect on uterine contractility in an unblinded and uncontrolled open-label first-in-human study in primary dysmenorrhea (Böttcher et al., 2014). On the other hand, Cirillo et al. (2007) reported the arrest of preterm labor in rodent models by the nonprostanoid and selective small-molecule FP antagonist AS604872, [(2S)-3-(4-phenylphenyl)sulfonyl-N-[(R)-phenyl(pyridin-2-yl)methyl]-1,3-thiazolidine-2-carboxamide].

We identified prodrug OBE022 [International Union of Pure and Applied Chemistry chemical name: (*S*)-2-amino-3-methyl-butyric acid (*S*)-3-{[(S)-3-(biphenyl-4-sulfonyl)-thiazolidine-2-carbonyl]-amino}-3-(4-fluoro-phenyl)-propyl ester, hydrochloride] and its parent OBE002 [International Union of Pure and Applied Chemistry chemical name: (*S*)-3-(biphenyl-4-sulfonyl)-thiazolidine-2-carboxylic acid [(S)-1-(4-fluoro-phenyl)-3-hydroxy-propyl]-amide] as orally active, potent, and highly selective FP receptor antagonists. We examined the tocolytic effects of OBE022 and/or OBE002 alone and in combination with nifedipine and atosiban in models of uterine contraction and preterm labor and assessed the risk on fetal development (including effects on the fetal kidneys, ductus arteriosus, and platelet aggregation) in comparison with indomethacin.

Materials and Methods

Drugs and Reagents. OBE022 and OBE002 were provided by ObsEva SA (Geneva, Switzerland). Dulbecco's modified Eagle's medium F-12 cell culture medium (DMEM-F-12) and inositol-free DMEM-F-12 were supplied by Thermo Fisher Scientific (cat. no. 11320-074; Paisley, UK; not listed). Atosiban and indomethacin were purchased from Sigma-Aldrich (cat. nos. A3480 and I7378; St. Quentin Fallavier, France). Dimethylsulfoxide (DMSO) was purchased from Sigma-Aldrich (cat. no. D2650; Dorset, UK). PGE_2 was from Tocris Bioscience (cat. no. 4027/1; Bristol, UK), and $PGF_{2\alpha}$ was from Cayman Chemicals (cat. no. 10007224; Ann Arbor, MI). Oxytocin (OT; Syntocinon) was purchased from Novartis Pharmaceuticals UK Ltd. (Camberley, UK). Vehicle for OT was Krebs solution, and OBE002 was dissolved in DMSO. OBE002 was diluted from stock solutions using DMSO shortly prior to the treatments. The DMSO concentration was adjusted to 0.1% (v/v) in all dose formulations, and control wells for OBE002 experiments were treated to contain 0.1% (v/v) DMSO. Saline was from B-Braun (Lapalisse, France).

Nifedipine and NP3S constituents 5% *N*-methylpyrrolidone, 30% polyethylene glycol 400, 25% polyethylene glycol 200, 20% propylene glycol, and 20% saline were purchased from Sigma-Aldrich (cat. nos. N7634, 494496, 8.07483, and 8.17003). A fresh solution of nifedipine was prepared in NP3S at a final concentration of 1 mg/ml (w/v). RU486 (mifepristone; [(8S,11R,13S,14S,17S)-11-[4-(dimethylamino)phenyl]-17-hydroxy-13-methyl-17-prop-1-ynyl-1,2,6,7,8,11,12,14,15,16-decahydrocyclopenta[a]phenanthren-3-one]) was purchased from Think Chemical (Hang Zhou, China). A fresh solution of RU486 was prepared at a final concentration of 0.25 mg/ml (w/v) in sesame oil (cat. no. S3547; Sigma-Aldrich) until a homogeneous suspension was observed.

Molecular Pharmacology Assays. Competitive displacement binding assays for human and rat FP receptors were performed in 10 mM 2-(N-Morpholino)ethanesulfonic acid/potassium hydroxide, 10 mM $MgCl_2$, 1 mM EDTA, pH 6.2 in the presence of 1 nM ^3H-$PGF_{2\alpha}$ (200 Ci/mmol, K_D = 1 nM; GE Healthcare, Chicago, IL) and 10–20 μg/well of membranes from HEK293-EBNA cells. Membranes from HEK293-EBNA cells stably transfected with FP receptors were prepared as described by Cirillo et al. (2007). All binding incubations

were done in a volume of 100 μl in Corning NBS 96-well plates (Corning, NY). Incubation time was 2 hours. Nonspecific binding was determined in the presence of 1 μM $PGF_{2\alpha}$. Wheatgerm Agglutinin SPA bead technology (RPNQ0001; GE Healthcare) was used for the FP receptor binding assays. K_i was calculated from IC_{50} using the Cheng-Prusoff equation: $K_i = IC_{50}/1 + ([L]/K_d)$, where [L] is the concentration of free radioligand used in the assay, and K_d is the dissociation constant of the radioligand for the receptor.

Agonist and antagonist effects of OBE022 and OBE002 in FP, EP_1, EP_2, EP_3, EP_4, prostaglandin D2 receptor 1, prostacyclin receptor, and thromboxane A2 receptors in cellular functional assays were determined at Eurofins Cerep (Celle L'Evescault, France). Conditions used were as follows (receptor/source/stimulus/measured component/detection method): DP_1/human recombinant Chinese hamster ovary (CHO) cells/BW245C/cAMP/ homogeneous time-resolved fluorescence (HTRF) (Degorce et al., 2009); EP_1/human recombinant HEK293 cells/PGE_2/intracellular Ca^{2+}/fluorimetry; EP_2/human recombinant CHO cells/PGE_2/cAMP/HTRF; EP_3/human recombinant HEK293 cells/sulprostone/impedance/cellular dielectric spectroscopy; EP_4/human recombinant CHO cells/PGE_2/cAMP/HTRF; FP/human recombinant HEK293 cells/$PGF_{2\alpha}$/intracellular Ca^{2+}/fluorimetry; IP/human recombinant CHO cells/iloprost/cAMP/HTRF; TP/human recombinant HEK293 cells/U44069/intracellular Ca^{2+}/fluorimetry.

Measurement of total inositol phosphates was made in HEK293-EBNA-FP cells labeled with *myo*-[3H] inositol. Confluent cells were incubated in DMEM-F-12 inositol-free medium containing 1 μCi/ml of *myo*-^{3}H-inositol (22 Ci/mmol, NET-114; PerkinElmer, Waltham, MA) for 24 hours at 37°C. Then, cells were rinsed once with DMEM-F-12 medium containing 10 mM LiCl, and compounds (agonist and/or antagonist) were added to the cells in the same medium for 60 minutes at 37°C in presence of 1% dimethylsulfoxide. The reaction was stopped by addition of 0.1 M formic acid, and cell lysates were loaded on Poly-Prep Chromatography columns (Bio-Rad, Hercules, CA) to separate radiolabeled components. After two washes with water, total ^{3}H-inositol phosphates were eluted with a solution containing 1 M ammonium formate and 0.1 M formic acid and determined by scintillation counting in a beta-counter. Specific total ^{3}H-inositol phosphate production was calculated by subtracting nonspecific production measured in the absence of agonist stimulation.

Uterine Contraction Model in Pregnant Human Myometrial Tissue. Myometrial tissues were collected from pregnant women undergoing scheduled elective caesarean section at term (38^{+0}–40 weeks of pregnancy) prior to the onset of labor. All participating women were informed about the nature of the study in advance, and informed written consents were provided with the approval from the local research ethics committee (Ethics No. RREC 1997-5089). Women with multiple pregnancies or medical conditions such as diabetes, pre-eclampsia, or obstetric cholestasis were not included in this study.

The myometrial biopsies were obtained from the upper margin of the incision made at the lower segment of the uterus, and were stored in phosphate-buffered saline at 4°C for dissection. All samples were used for contractility experiments within 24 hours of collection. The biopsies were dissected into eight longitudinal myometrial strips of $7 \times 2 \times 1$ mm and mounted to thermostatically controlled isolated organ baths (DMT Myograph 800MS; Danish Myo Technology A/S, Aarhus, Denmark) containing 7 ml of oxygenated (95% O_2 and 5% CO_2) Krebs solution [D-glucose 2.0 g/l, magnesium sulfate (anhydrous) 0.141 g/l, potassium phosphate monobasic 0.16 g/l, potassium chloride 0.35 g/l, sodium chloride 6.9 g/l, calcium chloride dihydrate 0.373 g/l, sodium bicarbonate 2.1 g/l] at 37°C, pH 7.4.

The longitudinal myometrial strips were subjected to 4*g* (19.62 mN) of tension to attain spontaneous contractions, and the experiment was abandoned if more than two strips failed to initiate stable spontaneous contractions. After recording 20 minutes of stable basal contractions, OBE002 (6, 60, 600, 6000 nM) or vehicle was added. The effect of OBE002 or vehicle was recorded for 10 minutes prior to cumulative dose responses for agonists (OT and $PGF_{2\alpha}$) that were added every 10 minutes. OT concentrations ranged from 1 to 100 nM, and $PGF_{2\alpha}$ concentrations ranged from 10 to 1000 nM. Myometrial contractility was recorded by a force transducer with Powerlab and were analyzed using LabChart 5 with peak parameters extension (version 5.5.6; ADInstruments, Oxford, UK). Acquired data were transferred from the data pad of the LabChart 5 software for further analysis. The changes in the contractility in response to different treatments were measured by normalizing to the basal spontaneous contractions of each strip and then to the equivalent time point for the vehicle control strip.

To assess potential additive or synergic effects of combined administration of OBE002 and other tocolytic compounds, such as the oxytocin receptor antagonist atosiban and the calcium channel blocker nifedipine, on OT-induced contractions, suboptimal concentrations of both compounds were determined (data not shown). For both compounds, a concentration of 6 nM was used to perform combination experiments using the methods described earlier.

Human Platelet Aggregation In Vitro Assay. The in vitro study was performed at Biotrial (Rennes, France). Human blood from five donors (one male, four female; nonsmokers; no concomitant drugs during the 10 days before collection; provided by Biotrial) was used to assess OBE002 and OBE022 inhibition of in vitro human platelet aggregation induced by arachidonic acid and collagen. Blood samples were centrifuged (200*g*; 10 minutes at room temperature), and platelet-rich plasma (PRP) was harvested using a 1-ml automatic pipette. Platelet concentrations were determined using a cell counter. The remaining blood was again centrifuged (2500*g*; 10 minutes at room temperature), platelet-poor plasma was collected, and platelet counts were performed. PRP was then adjusted to a platelet count of 250 G/L. OBE022 and OBE002 solutions with phosphate-buffered saline/DMSO were prepared to obtain PRP preparations containing 300/100/30/10/3/1 μM and 0.1% DMSO. Concentration ranges for indomethacin and aspirin were 100–0.1 and 300–0.3 μM, respectively. The aggregation assay was then performed using an eight-channel BioData PAP-8 platelet aggregometer (BioData Corp., Horsham, PA). Each reaction was monitored with a 430-nm light-emitting diode UV light source at a stir speed of 1100 rpm. To induce coagulation, arachidonic acid (1 mM) or collagen (2 μg/ml) was used. The extent of aggregation is defined as the maximal amount of light transmission reached ≤6 minutes after addition of the inducer [maximum platelet aggregation (MPA)] and the value of aggregation measured at 6 minutes after addition of the inducer [final platelet aggregation (FPA)]. The results are expressed as % MPA and % FPA, calculated relative to a baseline signal of 0% light transmission aggregation assay control (PRP) and 100% light transmission aggregation assay control (platelet-poor plasma), respectively. The percentage inhibition of aggregation (% IPA) was calculated as:

$$\%\ \mathrm{IPA}(M)x = ((\mathrm{MPA0} - \mathrm{MPA}x)/\mathrm{MPA0}) \times 100$$
$$\%\ \mathrm{IPA}(F)x = ((\mathrm{FPA0} - \mathrm{FPA}x)/\mathrm{FPA0}) \times 100$$

where $\mathrm{IPA}(M)_x$ and $\mathrm{IPA}(F)_x$ represent maximal and final IPA, respectively, in the presence of the test compound concentration x (between 0.3 and 300 μM). MPA_0 and FPA_0, and MPA and FPA represent maximal and final platelet aggregation measured at baseline (in the absence of test compounds) and in the presence of compound at concentration x (between 0.3 and 300 μM), respectively.

Animal Studies. All animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health or complied with national and European animal health directives, and were approved by the Institution's Animal Care and Use Committee or local equivalent. There were differences in the design of animal studies and the numbers of animals which stem from different reasons: the studies were performed in multiple laboratories over a prolonged period of time, different animal species (rat, mouse, rabbit) were used, and the specificities and technical difficulties inherent to each model led occasionally to animals not being suitable to undergo assessment (notably for the newborn rabbit model). However, each

single model was validated for the specific study purpose, and the numbers of animals and controls were comparable to those of published data and supported the observed results.

Spontaneous Uterine Contractions in Anesthetized Late-Term Pregnant Rats. Experimental conditions described by Cirillo et al. (2003, 2007) were used. In brief, late-term pregnant (gestational days 19–21) female Sprague-Dawley CD BR rats (Charles River, Calco, Italy), weighing 350–400 g, were anesthetized with urethane. One pregnant uterine horn was exposed, and a polyethylene catheter bearing on the tip a latex balloon filled with saline was inserted into the lumen. The catheter was connected to an amplifying and recording system via a pressure transducer. Increasing doses of OBE022 or OBE002 were orally administered (10, 30, and 60 mg/kg in NP3S) or injected by a 10-minute i.v. infusion (0.3, 1.0, and 3.0 mg/kg/min in NP3S). Groups of six rats per dose were used, and control groups comprised at least three rats. For the i.v. administration, the uterine contractile activity was quantified by calculating the area under the curve (AUC) during the 10-minute injection period. The percentage variation of the AUC values relative to the spontaneous uterine response observed after each compound administration was calculated in comparison with the value recorded before the first dose administration (basal value). The effect of OBE022 or OBE002 was evaluated by comparing pre- and post-treatment luminal uterine pressure values. For the oral administration, the same computation procedure was applied at different time points after treatment.

Mouse Preterm Parturition Model. This study was performed at Urosphere (Toulouse, France). At the beginning of the experiments, primigravid CD1 mice (Charles River Laboratories, Écully, France) on day 17 of pregnancy (about 85% gestation) were used. Mice were housed in groups of five under standard laboratory conditions, i.e., in an environmentally controlled room, acclimatized to a 12-hour light-dark cycle, and maintained on commercial solid food and tap water ad libitum.

Approximately 3 hours before induction of preterm labor [day 1 (D1) at 10am], the pregnant mice at gestational day 17 were placed in individual cages with food and water ad libitum Supplemental Fig. 1. On D1, pregnant mice received (at day time: 1pm) a single s.c. injection of RU486 at a dose of 2.5 mg/kg in a final volume of 10 ml/kg of sesame oil. OBE022 (10, 30, and 100 mg/kg) or nifedipine (5 mg/kg) was administered orally (p.o.) at a volume of 5 ml/kg once on D1 (6pm), twice on D2 (8am and 6pm), and once on D3 (8am) for a total of four administrations. For combination treatment, mice received OBE022 plus nifedipine using the same experimental design as single treatment.

From the first treatment (D1, around 5pm) onward, continuous monitoring using individual infrared HDCVI cameras (Dahua, Créteil, France) and a digital video recorder (Dahua) was performed to identify the time of the first pup's delivery and to record the number of live/dead pups. Since some of the pups may have died during the time elapsing between our inspections, all dead pups were controlled by the galenic hydrostatic pulmonary docimasy on whether they were born alive or dead: dead pups were dissected and a portion of the lung was placed in water. Lung floating over the water indicated air content and the ability of the pup to breath after birth (i.e., born alive). When the galenic hydrostatic pulmonary docimasy could not be assessed on a dead pup (no lung tissue available), it was considered born dead. At the end of the experiment, mice were euthanized by cervical dislocation.

Ductus Arteriosus Fetal Rat Model. The study was conducted at Citoxlab (Evreux, France). Wild-type female Han Wistar rats (10–11 weeks old; Charles River) were received at the test facility on gestation days 10–12. The rats were housed individually under standard laboratory conditions. During this acclimation period, 15 females were allocated randomly to three treatment groups (vehicle [polyethylene glycol 400 (PEG400)]/indomethacin/OBE002). Each animal was checked at least daily for clinical signs, and body weights were measured on days 12 and 21 of gestation.

Indomethacin was suspended in water containing 5% (w/v) arabic gum and administered to the rats through an orogastric tube. PEG400 was used as the vehicle for OBE002. OBE002 was dissolved in PEG400 and administered intravenously. All formulations were prepared extemporaneously and used within 2 hours after preparation.

Treatments were performed near term (mean gestational period: 21.5 days) on gestation day 21. Every five rats per treatment group received either an intravenous injection of PEG400 (0.8 ml/kg) or OBE002 (20 mg/kg, 0.8 ml/kg) or an oral administration of indomethacin (10 mg/kg in 5 ml/kg). Previous pharmacokinetic studies using the OBE002/PEG400 formulation resulted in exposures which were considered to be in large excess to those in anticipated clinical settings (ObsEva, data on file). The indomethacin dose level was selected on the basis of the ductus arteriosus (DA) assessments in rat fetuses previously published by Reese et al. (2006) and Toyoshima et al. (2006), which showed premature DA constriction.

The in situ morphology of the fetal DA was studied by direct macroscopic examination. Following administration of each test article to the dam, the fetuses were delivered 4 hours later by hysterectomy; dams were euthanized by inhalation of carbon dioxide gas followed by cervical dislocation. Immediately after hysterectomy, each live fetus was euthanized by an intraperitoneal injection of sodium pentobarbital, and its body weight was measured. Within less than 1 hour after euthanasia, approximately half of the live fetuses in each litter (3–7) were subjected to macroscopic examination of the DA and then kept preserved in a fixative for potential microscopic examination. The thorax was opened by midline incision. Constriction of the DA was evaluated under a binocular microscope using the following scoring system: no constriction (0), one-third of the DA is constricted (1), two-thirds of the DA is constricted (2), all of the DA is constricted (3). Schematic drawings of the grading system are presented in Supplemental Fig. S2. Photographs of the frontal plane of each examined fetus were taken.

In addition, microscopic examinations were performed in selected animals using a methodology similar to Reese et al. (2006). In brief, serial sections through the fetal and newborn thorax allowed quantitative assessment of DA diameter in relation to the size of the transverse aorta. The inner diameters of the DA and aorta were measured, and the size of the DA was expressed as a percentage of the diameter of the transverse aortic arch (ratio of ductus arteriosus to aorta).

Renal Function Newborn Rabbit Model. This study was performed at Porsolt Research Laboratory (Le Genest-Saint-Isle, France). Pregnant New Zealand White rabbits were purchased from CEGAV (Saint Mars d'Egrenne, France) or HYPHARM (Sèvremoine, France) and maintained in single housing until delivery under standard laboratory conditions. Thirty-five newborn New Zealand White rabbits from eight litters, 5–8 days old (mean ± S.E.M.: 7.0 ± 0.2 days) with a body weight of 138.4 ± 8.5 g were studied. All animals were born by spontaneous vaginal delivery and breastfed until the time of study. The methods used in this study were similar to those described by Chamaa et al. (2000) and Prévot et al. (2004).

The newborn rabbits were assigned to three groups of comparable age and body weight. Thirteen newborn rabbits were used in each indomethacin and OBE002 treatment group and nine in the vehicle control group. To set up the experiment, the rabbits were anesthetized (40 mg/kg sodium pentobarbital s.c., followed by subsequent i.p. doses when needed) and ventilated (rodent ventilator model 683; Harvard Apparatus, Holliston, MA) via tracheostomy with an oxygen-enriched gas (approximately 32% O_2). The respiratory rate was kept at 40 breaths/min with the tidal volume adjusted for age and body weight. The body temperature, recorded by a rectal probe (model BAT-12; Physitemp Instruments Inc., Clifton, NJ), was kept at approximately 39°C by using a heating table and infrared lamps. Polyethylene catheters were implanted in a jugular vein for dosing and in a carotid artery for blood sampling and for continuous monitoring of mean arterial blood pressure and heart rate [Bridge Amp Model FE221 (ADInstruments, Sydney, Australia); data acquisition system and software: PowerLab Model ML870, LabChart 7 Pro, version 7.3.5 (ADInstruments Ltd., Paris, France)]. The bladder was catheterized through the urethra for continuous urine collection.

After completion of the surgical procedure, the animals received a slow i.v. bolus of inulin (IN) and para-aminohippurate (PAH) at 100 and 1.25 mg/kg, respectively, followed by a continuous infusion at a rate of 1 mL/100 g body weight/h of a solution containing (per liter) 50 g of mannitol, 3 g of IN, 0.15 g of PAH, 150 mmol of sodium, 105 mmol of chloride, 5 mmol of potassium, and 50 mmol of sodium bicarbonate. This enabled the determination of the inulin and PAH clearances (C_{IN} and C_{PAH}), representing glomerular filtration rate (GFR) and renal plasma flow (RPF), respectively, throughout the experiment.

Once the study setup was completed, the animals had a 90-minute equilibration period, followed by a 60-minute control period consisting of two 30-minute urine collections with 0.4 ml of blood withdrawn at the midpoint of each collection. Thereafter, the rabbits received a slow i.v. bolus injection of the vehicle, indomethacin, or OBE002. Urine was collected for an additional 60-minute period postadministration (test period consisting of two 30-minute urine collections with 0.4 ml of blood withdrawn at the midpoint of each).

One hundred microliters of blood was used for immediate measurements of blood gases, pH, and hematocrit. After centrifugation, the remaining plasma was stored frozen for later determinations of plasma protein, inulin, and PAH concentrations. Following plasma collection, red blood cells were reconstituted in a modified Ringer solution and reinfused to the newborn rabbit. Urine samples were stored frozen until analyses for inulin and PAH concentrations were performed. All animals were euthanized after the last scheduled assessment.

The indomethacin dose of 2 mg/kg i.v. in PEG400 was chosen based on previously published experiences with this rabbit model. Duarte-Silva et al. (1986) reported intense vasoconstriction in the developing rabbit kidney. The dose selected for OBE002 was 10 mg/kg i.v. in PEG400. This dose was tested in pharmacokinetic studies in New Zealand White rabbits and resulted in peak exposures exceeding estimated human exposures multiple times (anticipated to be administered by the oral route) (ObsEva, data on file).

Urine volume was assessed gravimetrically to calculate urinary flow rate (UFR). Blood gas determinations were performed using a pH/blood gas analyzer (VetScan i-STAT; Abaxis Veterinary Diagnostics, Union City, CA). Plasma protein concentrations were measured by direct potentiometry (Konelab 20i; Thermo Fisher Scientific). Concentrations of inulin and PAH were determined in urine and plasma samples using a colorimetric anthrone method for inulin and the colorimetric Bratton and Marshall's method for PAH, respectively. Inulin and PAH clearances, representing GFR and RPF, renal blood flow (RBF), filtration fraction (FF), and renal vascular resistance (RVR), were derived from standard equations as previously reported by Chamaa et al. (2000). For the calculation of RPF, a correction factor of 0.55 was used. The latter was previously established in this rabbit model by Chamaa et al. (2000).

Data Analysis. For contractions in human myometrial tissue, all results were expressed as the mean ± S.E.M., with $n \geq 6$ experiments performed on myometrial strips from different patients. Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA). Two-way analysis of variance or analysis of variance of repeated measures was conducted with Bonferroni post-hoc test. Values were considered to be statistically significant at $P < 0.05$.

For the mouse preterm parturition model, statistical analyses were performed using GraphPad Prism (GraphPad Software Inc.). A P value <0.05 was accepted for statistical significance. Before carrying out any statistical test, the data were tested for normal distribution (Shapiro-Wilk normality test) and their variance evaluated (F test). Consequently, the appropriate statistical test was applied. All statistical tests were considered to be exploratory and no adjustment for multiplicity was performed. For mean time of first pups' delivery and percentage of living pups and comparison between two experimental groups, unpaired t test was used as a parametric test, unpaired t test with Welch's correction was used as an unequal variance parametric test, and Mann-Whitney test was used as a nonparametric t test. For percentage of delivery, comparison between two experimental groups was performed with a log-rank (Mantel-Cox) test.

In the ductus arteriosus fetal rat model, results were expressed as means ± S.D. Macroscopic intergroup comparisons were performed using a one-way analysis of variance (ANOVA) with "group" as a factor, followed by Tukey's multiple comparisons test in case of a significant effect.

In the renal function newborn rabbit model, all parameters are reported for the control, pretest period (mean value of the two 30-minute periods predose) and for the test period (mean value of the two 30-minute postdose periods). Results are presented as the mean ± S.E.M.; median and interquartile ranges were also calculated. To reduce potential bias due to interindividual variability, intergroup comparisons were exclusively performed on individual percentage changes from pretest (mean values of the two test periods vs. mean values of the two pretest periods) using a one-way ANOVA with "group" as a factor, followed by Tukey's multiple comparisons test in case of a significant effect. The presented results include all available data and were carried out using formulae and correction factors previously reported for this model to generate comparable results. For some animals, this approach produced data which was outside expected biologic ranges (i.e., FF > 100%; outliers).

For the in vitro platelet aggregation study, % MPA and % FPA, in relation to inhibitor concentration, were analyzed using a curve-fitting program (GraphPad Prism; GraphPad Software Inc.), and concentration needed for half-maximal inhibition was calculated for each compound. The resulting IC_{50} values of OBE002 and OBE022 were compared with those of aspirin and indomethacin.

In the pregnant rat model of spontaneous contractions, statistical differences between treatment groups at each time point were determined by using one-way ANOVA followed by Tukey test. Values were considered to be statistically significant at $P < 0.05$.

Results

Molecular Pharmacological Characterization of Potent and Selective FP Receptor Antagonists. OBE022 and OBE002 were discovered by optimization, using medicinal chemistry principles, of a hit compound originally identified by screening a large collection of compounds against the FP receptor in a radioligand binding assay using ^{3}H-$PGF_{2\alpha}$ and recombinant HEK293 cells stably transfected with human FP. Compound OBE022 is the valine ester prodrug of OBE002 (Fig. 1). Pharmacokinetic studies in rats and dogs showed that OBE022 has higher oral bioavailability than OBE002 (ObsEva, data on file). Oral administration of OBE022 in rat or dog showed rapid, mostly presystemic, and extensive conversion into OBE002, with exposure levels of OBE002 up to 25-fold those of OBE022.

OBE022 and OBE002 were assayed for FP binding affinity by competitive binding analysis with ^{3}H-$PGF_{2\alpha}$ using HEK293 cells stably transfected with the FP receptor. Binding affinities (K_i) of OBE022 for the human and rat FP receptor were 1 and 26 nM, respectively. For OBE002, K_i values were 6 nM for the human and 313 nM for the rat FP receptor. The binding of both compounds was reversible and competitive since increasing concentrations of either compound caused successive decreases in the slope of the binding curves, consistent with an increase in equilibrium dissociation constant (K_D) without a reduction in receptor density (B_{max}) (data not shown).

OBE022 and OBE002 are highly selective antagonists for FP compared with the other human prostaglandin receptor subtypes when tested for antagonist or agonist effects in competitive displacement binding assays in cellular receptor

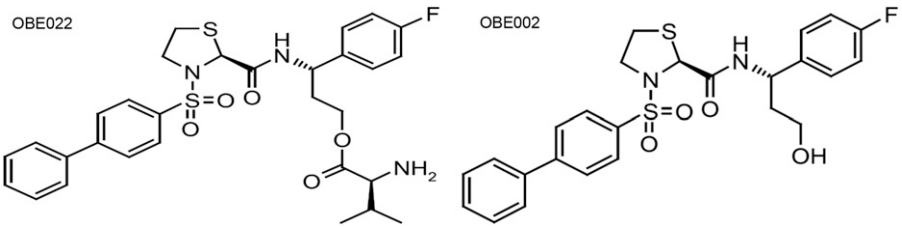

Fig. 1. Chemical structures of OBE022 prodrug and OBE002 parent compound.

assays (Table 1). The high selectivity profiles of OBE022 and OBE002 at 10 μM were further demonstrated in binding assays against a panel of 50 receptors (including the oxytocin receptor) and enzymes (data not shown). The functional characterization of OBE002 on human FP was also performed in transfected HEK293-EBNA cells. OBE002 was able to dose-dependently inhibit the synthesis of inositol triphosphates with an IC_{50} value of 60 nM. When added alone to the cells, OBE002 tested up to 10 μM did not induce any synthesis of inositol triphosphates, indicating that the compound is devoid of agonist activity.

Inhibition of $PGF_{2\alpha}$- and OT-Induced Contractions of Human Pregnant Myometrium In Vitro. $PGF_{2\alpha}$ stimulation consistently induced contractions of strips of human pregnant myometrium ex vivo (Fig. 2). There was a steady, dose-dependent increase in the overall contractile output, affecting the average area under the curve, peak amplitude, and duration. Incubation with OBE002 had a significant effect on the spontaneous contractility when compared with its vehicle control (Fig. 2). OBE002 antagonized the effects of $PGF_{2\alpha}$ upon the rate of contractions, average area under the curve, peak tension, and duration of the contractions (Fig. 2, A–D). The total work done–induced $PGF_{2\alpha}$ was inhibited by OBE002 (Fig. 2E).

Increasing doses of OT produced an increase in the rate of contractility, peak tension, and the average area under the curve in a dose-dependent manner, having an overall effect on the total work done. The stimulatory effect of OT was inhibited by using various concentrations of OBE002 (Fig. 3). OBE002 produced a concentration-dependent inhibition of OT-induced myometrial contractility, significantly reducing the rate of contractility, average area under the curve, and peak tension (Fig. 3, A–C). However, there was no apparent effect on the duration of contractions (Fig. 3D). The total work done induced–OT was inhibited by OBE002 by 59.9% compared with vehicle control (Fig. 3E).

The inhibitory effect of atosiban on OT-induced myometrial contractility was enhanced in the presence of OBE002; however, this did not reach significance when compared with OBE002 alone (Fig. 4). Treatment with nifedipine and OBE002 alone inhibited the OT-induced contractions, and their inhibitory effects were increased further when the nifedipine and OBE002 were used together compared with each drug alone (Fig. 5).

Inhibition of Spontaneous Uterine Contractions in the Pregnant Rat. Spontaneous uterine contractions were measured as AUC of contraction recording over time. This captures the overall effect on contraction work rate, including intensity, frequency, and length of single contractions (Cirillo et al., 2003, 2007). Both OBE022, given orally, and OBE002, administered i.v., markedly and dose-dependently reduced the spontaneous uterine contractions in late-term pregnant rats at gestational days 19–21 (Fig. 6). The inhibitory effect following the oral administration appeared with a fast onset and remained at sustained level up to the end of the observation period (Fig. 6B).

Effect of OBE022 and Nifedipine in a Mouse Preterm Parturition Model. Time course of the cumulative percentage of delivered mice after RU486-induced preterm parturition at gestational day 17 in OBE022, nifedipine, or vehicle treatment groups is presented in Fig. 7. Oral treatment with OBE022 delayed the preterm birth caused by RU486 administration as reflected by a shift to the right of the percentage of delivery curve (Fig. 7A). The effect of oral treatment with nifedipine was comparable. Both OBE022 and nifedipine showed a trend to increase the time of first pup delivery (Fig. 7B). As an important consequence of the prolongation of gestation, dams delivered viable pups.

Combination of OBE022 and nifedipine caused a synergistic effect on the delay of RU486-induced preterm birth as reflected by a more pronounced shift to the right of the percentage of delivery curve, in comparison with OBE022 or nifedipine alone. Also, a larger increase of the time of first pup delivery was observed (Fig. 7, A and B). No significant change was observed in percentage of living pups with OBE022 plus nifedipine combination in comparison with either vehicle group or each test substance alone (Fig. 7C).

Effects of OBE002 and Indomethacin on Renal Function in the Newborn Rabbit. The renal functional responses to i.v. indomethacin, OBE002, or vehicle are shown in Supplemental Table S1 (absolute data) and Fig. 8 (percentage changes). The baseline values for the measured and

TABLE 1

Equilibrium dissociation constants for OBE022 and OBE002 antagonists in competitive binding assays to human prostanoid receptors transfected in recombinant CHO or HEK293 cells

Results are expressed as K_B in nanomolar for antagonist effect. K_B values were calculated using the modified Cheng-Prusoff equation $K_B = IC_{50}/1 + ([A]/EC_{50A})$, where [A] is the concentration of the agonist used in the assay, and $EC_{50A} = EC_{50}$ value of the reference agonist. OBE022 and OBE002 showed no agonist effect in any assay.

Receptor	FP	EP_1	EP_2	EP_3	EP_4	DP_1	IP	TP
OBE022	5.9	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000
OBE002	5.4	>10,000	1600	>10,000	>10,000	>10,000	410	>10,000

DP, prostaglandin D2 receptor; IP, prostacyclin receptor; TP, thromboxane A2 receptor.

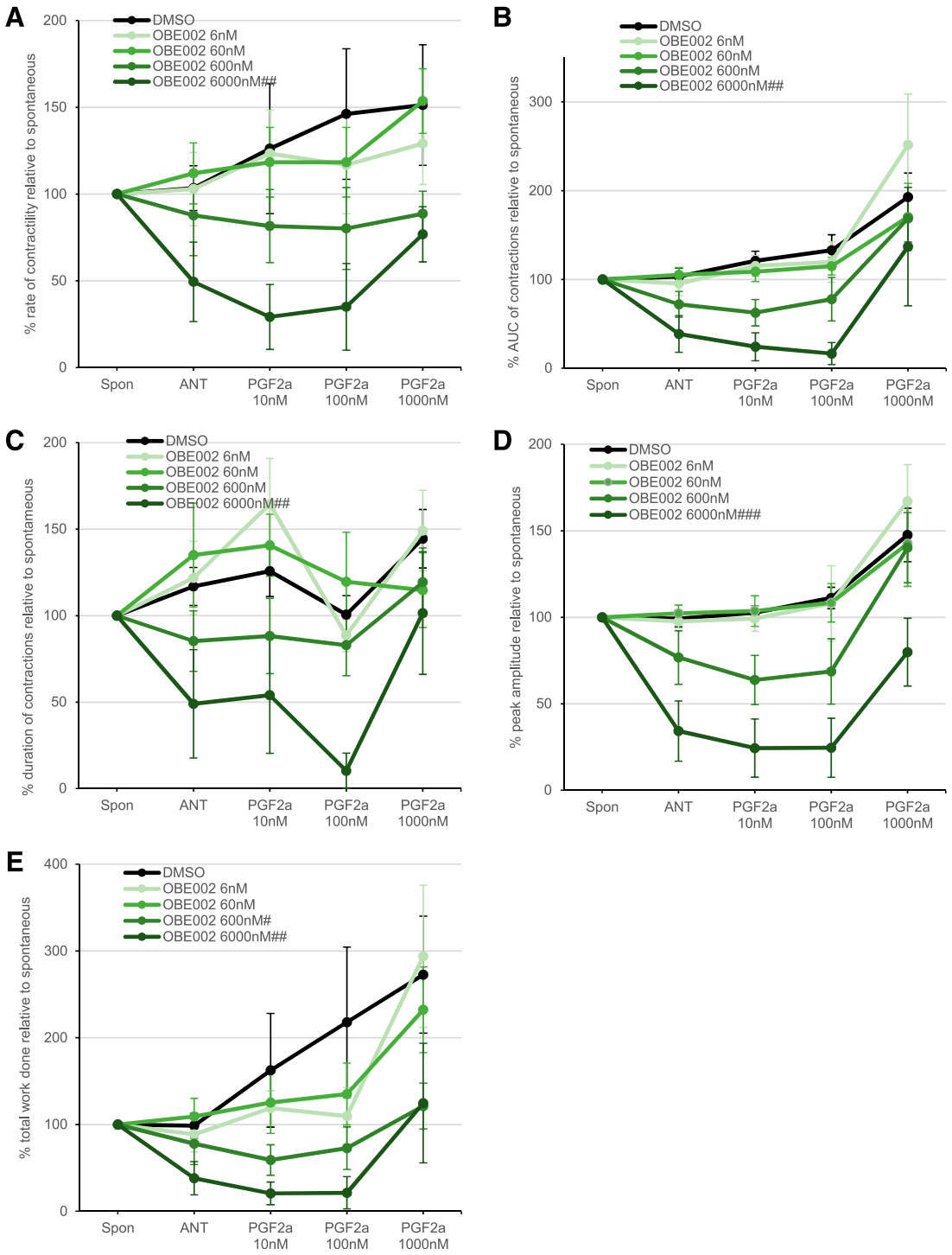

Fig. 2. The effect of OBE002 on spontaneous and $PGF_{2\alpha}$-induced human myometrial contractions. Prelabor lower segment myometrial biopsies were subjected to stretch force of 4*g* to attain spontaneous contractions. After 20 minutes of basal reading, vehicle control (DMSO) or OBE002 (6, 60, 600, or 6000 nM) was added, and its effect on spontaneous contractions was measured for 10 minutes. The effect of the OBE002 upon $PGF_{2\alpha}$ was then measured by adding increasing concentrations of the agonist (10, 100, and 1000 nM) at 10-minute intervals. For any individual strip, values for rate of contraction (A), work per contraction (AUC) (B), contraction duration (C), contraction peak (D), and total work (area under all contractions) (E) were measured for each experimental time point and re-expressed as a ratio to the baseline period measurements (n = 6 independent experiments; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ vs. DMSO; ANOVA). ANT, antagonist.

calculated renal functional parameters were generally comparable for all three animal groups. After the administration of indomethacin, UFR fell significantly, whereas slight to significant increases in UFR were noted in animals treated with OBE002 or vehicle, respectively (Supplemental Table S1); the pre-/post-treatment percentage difference of the indomethacin group was significantly lower than for animals treated with OBE002 or vehicle (Supplemental Table S2). Similar changes, although generally not statistically significant, were observed for GFR; only the pre-/post-treatment

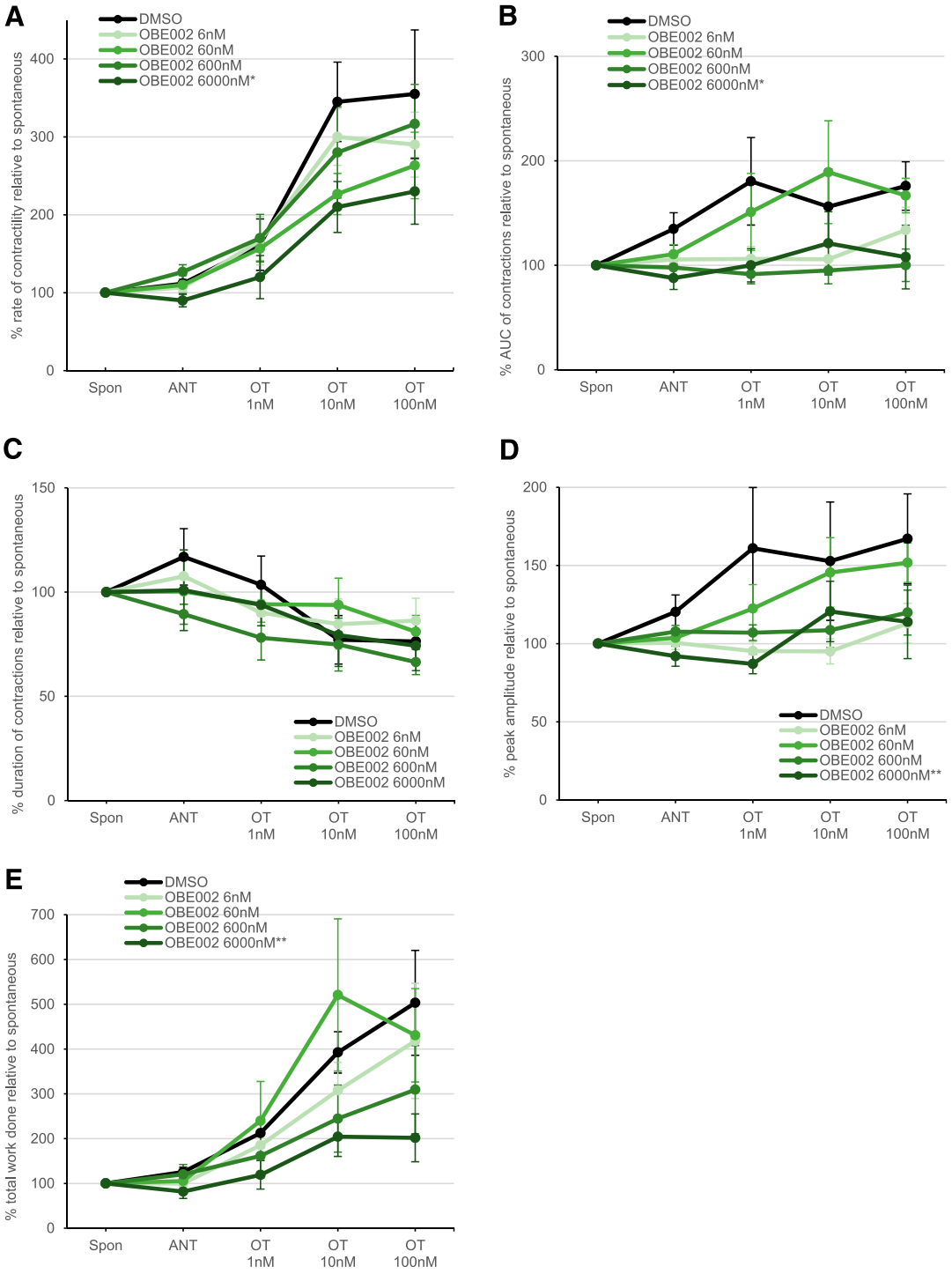

Fig. 3. The effect of OBE002 on spontaneous and OT-induced human myometrial contractions. Prelabor lower segment myometrial biopsies were subjected to stretch force of 4*g* to attain spontaneous contractions. After 20 minutes of basal reading, vehicle control (DMSO) or OBE002 (6, 60, 600, or 6000 nM) was added, and its effect on spontaneous contractions was measured for 10 minutes. The effect of OBE002 upon OT was then measured by adding increasing concentrations of agonist (1, 10, and 100 nM) at 10-minute intervals. For any individual strip, values for rate of contraction (A), work per contraction (AUC) (B), contraction duration (C), contraction peak (D), and total work (area under all contractions) (E) were measured for each experimental time point and re-expressed as a ratio to the baseline period measurements (n = 6 independent experiments; $^{*}P < 0.05$; $^{**}P < 0.01$ vs. DMSO; ANOVA). ANT, antagonist.

comparison within the indomethacin group showed a significant reduction (Supplemental Table S1). RPF and RBF showed similar trends: reduced values for the indomethacin group and increased values for OBE002 and vehicle, the latter reaching significance (Supplemental Table S1); marked variability was noted in single animals treated with indomethacin as indicated by the gap between mean and median percentage differences in this group (Supplemental Table S2). FF slightly decreased in all groups (Supplemental Table S2); single animals treated with OBE002 had

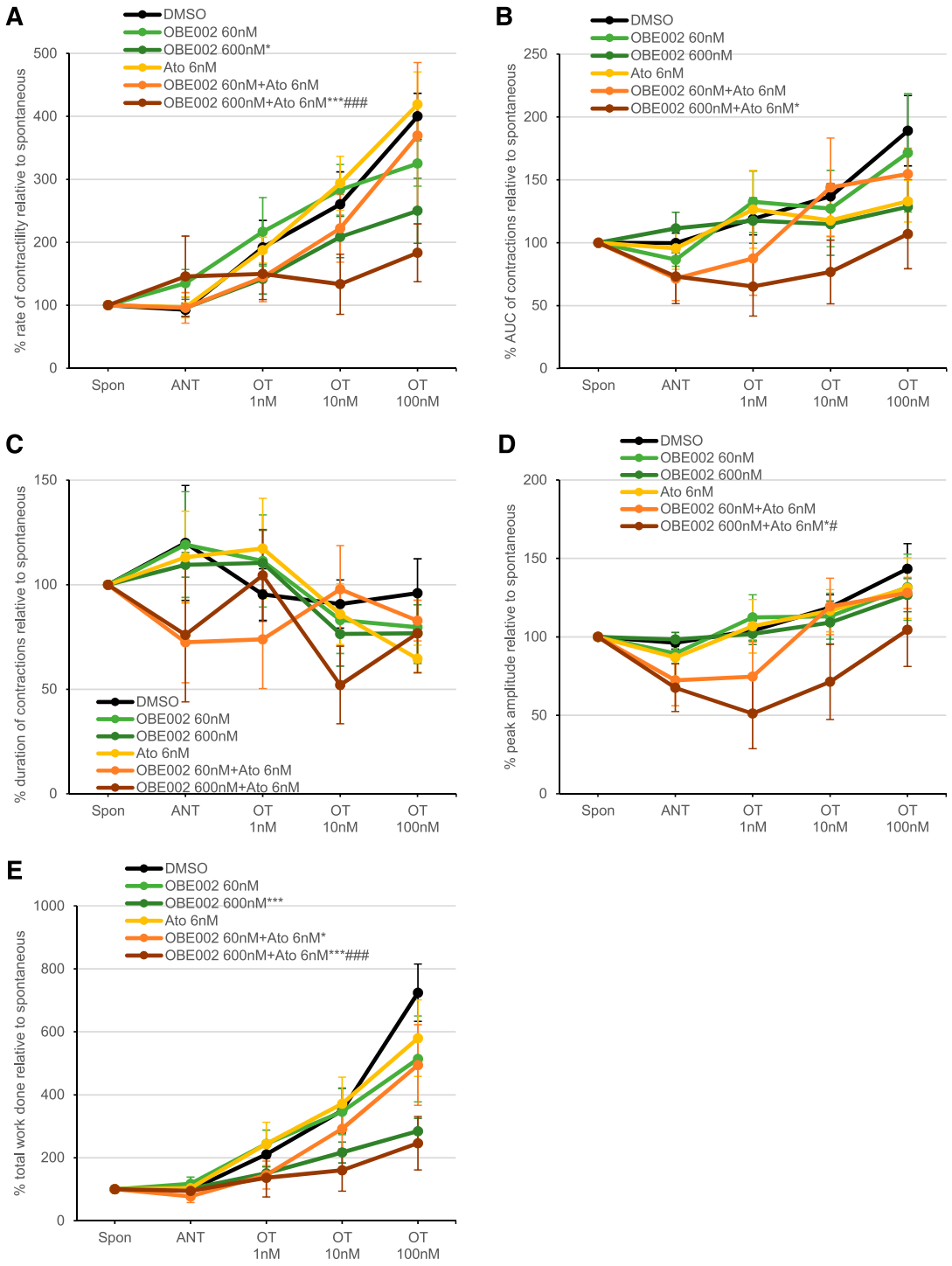

Fig. 4. The combination effect of OBE002 and atosiban on spontaneous and OT-induced human myometrial contractions. Prelabor lower segment myometrial biopsies were subjected to stretch force of 4*g* to attain spontaneous contractions. After 20 minutes of basal reading, vehicle control (DMSO) or atosiban (Ato, 6 nM) and OBE002 (60 or 600 nM) alone or in combination was added, and its effect on spontaneous contractions was measured for 10 minutes. The effect of the atosiban and OBE002 upon OT was then measured by adding increasing concentrations of agonist (1, 10, and 100 nM) at 10-minute intervals. For any individual strip, values for rate of contraction (A), work per contraction (AUC) (B), contraction duration (C), contraction peak (D), and total work (area under all contractions) (E) were measured for each experimental time point and re-expressed as a ratio to the baseline period measurements (n = 6 independent experiments; $^{*}P < 0.05$; $^{***}P < 0.001$ vs. DMSO; $^{\#}P < 0.05$; $^{\#\#\#}P < 0.001$ vs. Ato 6 nM; ANOVA). ANT, antagonist.

variable results, leading to an apparent increase in FF (Supplemental Table S1). As expected, RVR increased in the indomethacin group and decreased in the OBE002 and vehicle groups (Supplemental Table S2); single animals in the OBE002 group had outliers in RVR, which led to discordant mean and median percentage differences (Supplemental Table S2) and marked S.E.M. values (Supplemental Table S1).

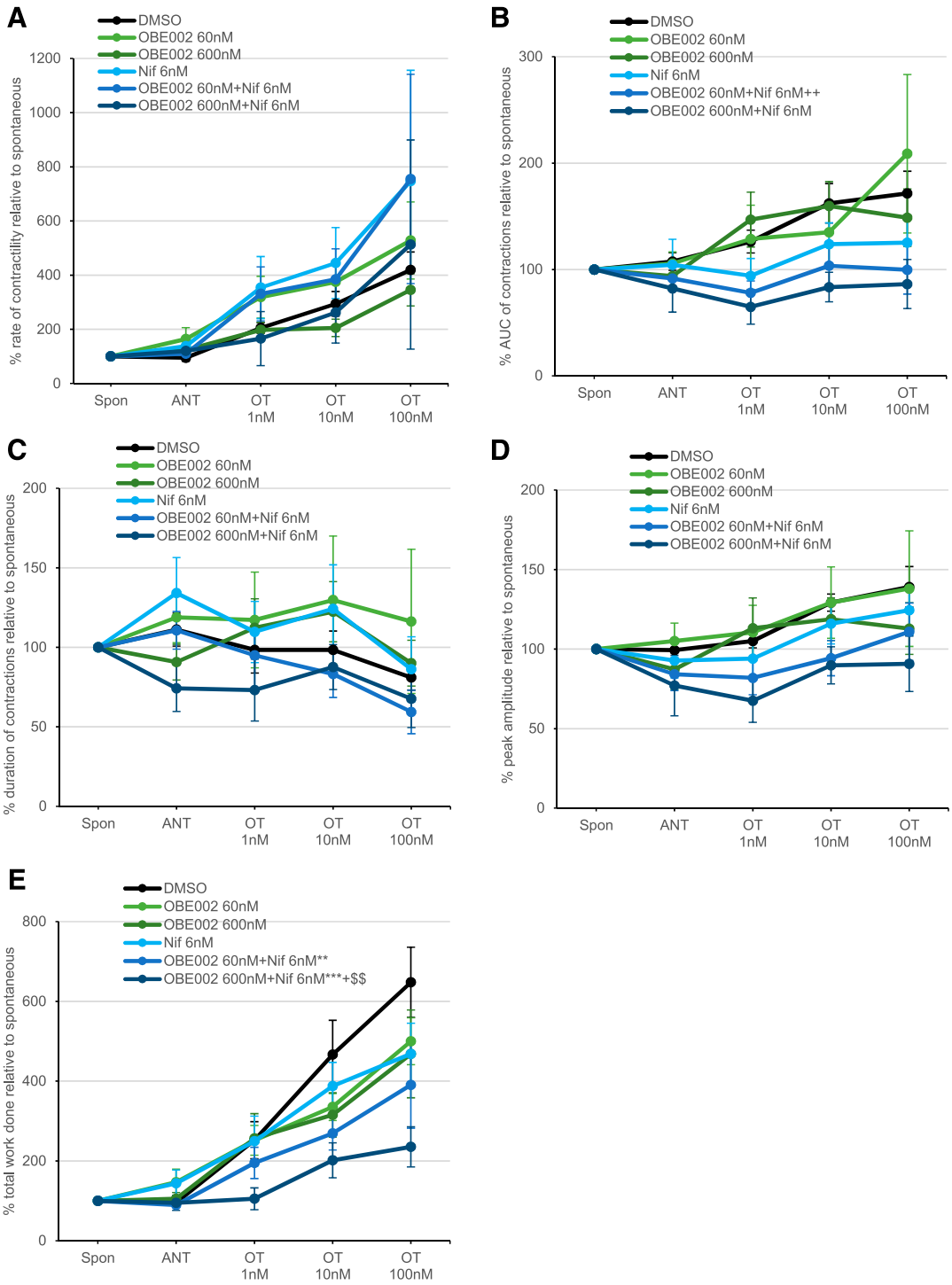

Fig. 5. The combination effect of OBE002 and nifedipine on spontaneous and OT-induced human myometrial contractions. Prelabor lower segment myometrial biopsies were subjected to stretch force of 4*g* to attain spontaneous contractions. After 20 minutes of basal reading, vehicle control (DMSO) or nifedipine (Nif, 6 nM) and OBE002 (60 or 600 nM) alone or in combination was added, and its effect on spontaneous contractions was measured for 10 minutes. The effect of the nifedipine and OBE002 upon OT was then measured by adding increasing concentrations of agonist (1, 10, and 100 nM) at 10-minute intervals. For any individual strip, values for rate of contraction (A), work per contraction (AUC) (B), contraction duration (C), contraction peak (D), and total work (area under all contractions) (E) were measured for each experimental time point and re-expressed as a ratio to the baseline period measurements (n = 8 independent experiments; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. DMSO; $^{+}P < 0.05$; $^{++}P < 0.01$ vs. OBE002; $^{\$\$}P < 0.01$ vs. Nif 6 nM; ANOVA). ANT, antagonist.

Supplemental Table S1 summarizes the variables indicating the general status of the newborn animals throughout the experiments. Mean blood pressure and $PaO_2/PaCO_2$ did not change significantly from the control values in any of the three animal groups. Heart rate slightly decreased after the administration of indomethacin, and pH slightly decreased after treatment in the indomethacin and vehicle groups; heart rate and pH changes remained in the range of values observed at

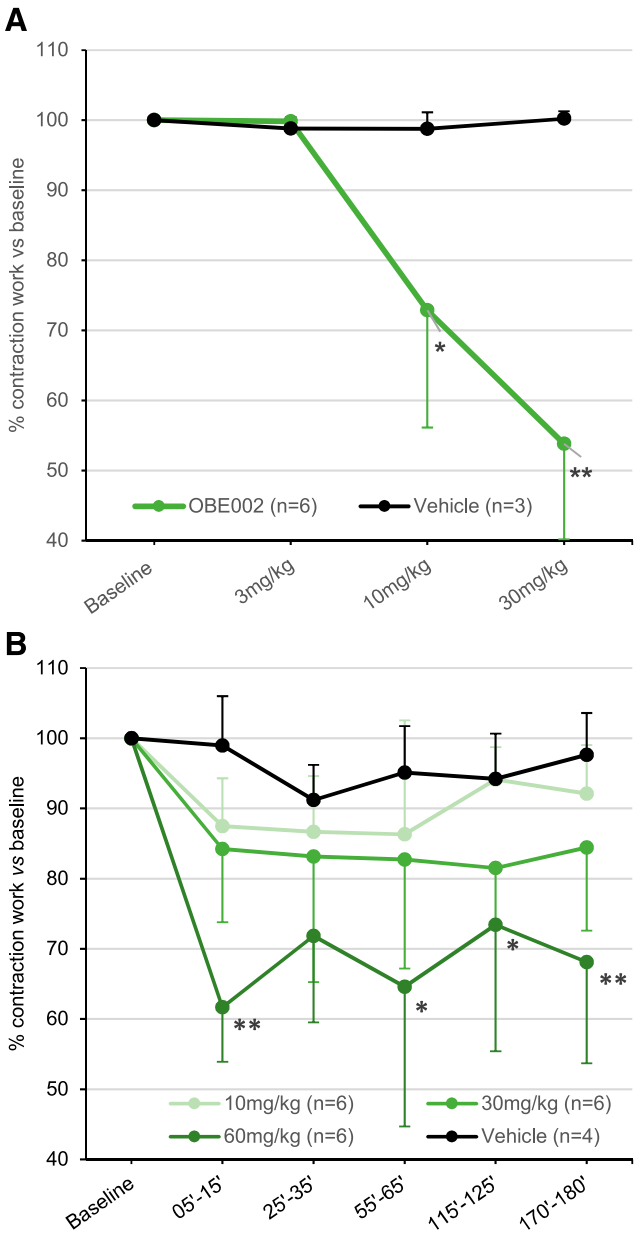

Fig. 6. Effect of OBE002 and OBE022 in reducing spontaneous contractile activity in pregnant rat near term. (A) Dose-related effects of OBE002 administered by i.v. infusion. The uterine contractile activity was quantified by calculating the AUC during the 10-minute injection period. (B) Time-dependent effects of OBE022 after a single oral administration at 10, 30, and 60 mg/kg. $n = 6$ animals per dose. *$P < 0.05$; **$P < 0.01$ vs. vehicle-treated group.

pretreatment in the OBE002 group. The hematocrit and plasma protein levels fell slightly, albeit significantly, in all three animal groups. These are recurrent findings in the neonatal animal experiments as a result of blood sampling (Prévot et al., 2004).

Effects of OBE002 and Indomethacin on Constriction of the Ductus Arteriosus in Fetal Rat. We compared the effects of intravenous OBE002 (20 mg/kg) and oral indomethacin (10 mg/kg) on DA constriction in a fetal rat model using an in situ macroscopic evaluation technique.

In the dams, there were no clinical signs and no relevant body weight differences between groups before treatment. All fetuses were alive and showed evidence of an intact circulation; there were no relevant differences in mean fetal body weights between groups.

Under macroscopic evaluation, fetuses of the PEG400 control group had no DA constriction (mean ± S.D.: 0.00 ± 0.00). For fetuses from the indomethacin group, 12 out of 24 fetuses from four out of four different litters had a constriction affecting one-third of the DA, and four out of 24 fetuses from three different litters had a constriction affecting two-third of the DA (mean ± S.D.: 0.83 ± 0.19). In the OBE002 group, one fetus had a constriction affecting one-third of the DA; all 28 others had no DA constriction (mean ± S.D.: 0.03 ± 0.18).

Microscopic examination of DA/artery aorta (DA/AA) ratios of microscopically examined animals correlated well with the corresponding macroscopic DA grade. For a DA grade of 0–(no constriction), we observed DA/AA ratios between 0.29 and 0.46 ($n = 8$), a DA grade of 1 (one-third of the DA constricted) had a DA/AA ratio of 0.25 ($n = 1$), and a DA grade of 2 (two-thirds of the DA constricted) was at a DA/AA ratio of 0.06–0.12 ($n = 3$). Examples of macroscopic DA constriction grades are presented in Supplemental Fig. S2.

Effect of OBE022 and OBE002 on Human Platelet Aggregation In Vitro by Light Transmission Aggregation. In vitro inhibition of human platelet aggregation induced by arachidonic acid and collagen was assessed. The effects OBE002 and OBE022 were compared with those of the pan-prostaglandin inhibitors aspirin and indomethacin. The IPA on the % MPA and on the % FPA were used to analyze the IC_{50}.

Figure 9 shows results when arachidonic acid was used to initiate aggregation; the IC_{50} values of aspirin for % MPA and % FPA were 4.1 and 4.0 μM, respectively. The IC_{50} of indomethacin could not be estimated since aggregation was inhibited beyond 50% at the lowest concentration (56.5% and 56.0% at 0.33 μM, respectively); thus, the IC_{50} of indomethacin is <0.33 μM. As for OBE022 and OBE002, the mean IPA for % MPA and % FPA at the highest feasible concentration (1000 μM) was between 12.7% and 16.8%, and thus, the IC_{50} of OBE022 and OBE002 is >1000 μM.

When collagen was used to initiate aggregation, the IC_{50} for % MPA and % FPA of aspirin could not be determined: % MPA and % FPA were 2.1% and 1.3% at 1.00 μM and plateaued at 46.1% and 47.5% at concentrations of 33.33 μM or higher, respectively. Similarly, % MPA and % FPA of indomethacin were 5.4% and 6.0% at 0.33 μM and plateaued at 47.5% and 48.6% at concentrations of 3.33 μM or higher, respectively. For OBE022 and OBE002, the mean IPA for % MPA and % FPA at 333.33 μM was below 10% and between 21.8% and 29.1% and at the highest feasible concentration (1000 μM).

Thus, OBE022 and OBE002 did not inhibit platelet aggregation initiated by arachidonic acid at concentrations >1000 μM ($IC_{50} > 1000$ μM), whereas significant inhibition of platelet aggregation was observed for indomethacin ($IC_{50} < 0.33$ μM) and aspirin ($IC_{50} = 4$ μM). OBE022 and OBE002 did not inhibit collagen-induced platelet aggregation, and due to the limitations of the assay, aspirin and indomethacin led only to partial inhibition at the highest concentration tested.

Discussion

We have designed the pro-drug OBE022, a new orally active small molecule, that is readily absorbed and converted into its parent OBE002. Both compounds are highly potent, selective,

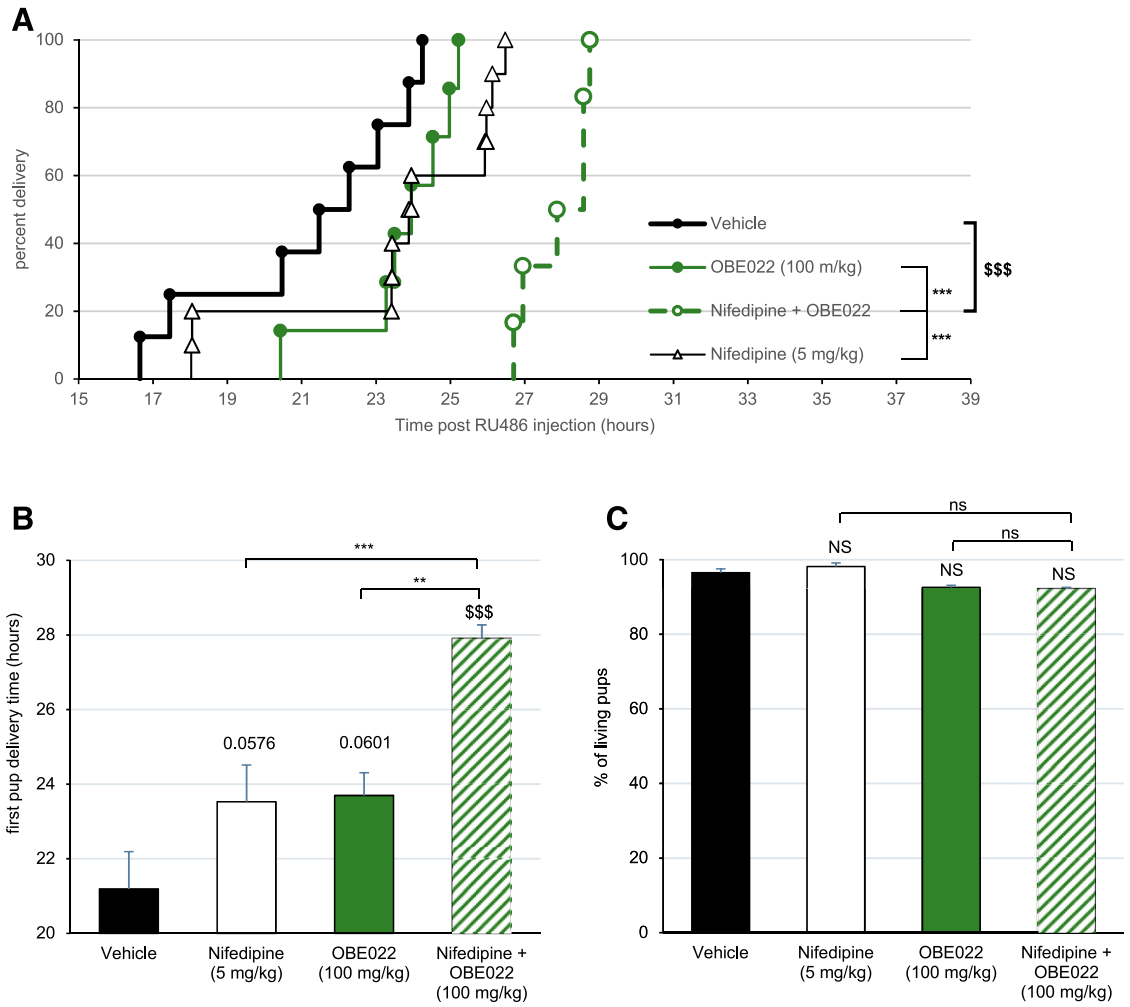

Fig. 7. Effects of OBE022 and nifedipine in the mouse model of RU486-induced preterm parturition. (A) Time-course of delivery expressed as a percentage of delivered animals after RU486 administration. (B) Mean time of first pup delivery. (C) Percentage of living pups. Results are expressed as the mean ± S.E.M. (n = 6–10 animals per group). $^{\$\$\$}P < 0.001$ vs. vehicle; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. combination, log rank (Mantel-Cox) test or Mann-Whitney. NS not significant vs. vehicle; ns not significant vs combination.

competitive, reversible antagonists of the FP receptor. Although OBE002 is the main circulating active molecule, the pharmacology of both molecules was characterized in vitro and in vivo. In isolated pregnant human myometrium smooth muscle strips, we demonstrated that OBE002 inhibits both $PGF_{2\alpha}$- and OT-induced contractions of pregnant human myometrium strips, suggesting possible cross-talk between FP and OT receptors. Similar to our results, allosteric FP receptor antagonist THG113.31 has been shown to inhibit OT-induced contractions in a dose-dependent manner (Friel et al., 2005), and the reverse was shown in term myometrial samples, as OT receptor antagonists atosiban and nolasiban were able to inhibit PG-induced contractions (Kim et al., 2017). Functional cross-talk between G protein–coupled receptors may occur via direct interaction between the receptors or through an interplay of downstream signaling pathways (Kamal and Jockers, 2011). Previous studies have indicated a potential cross-talk between FP and OT receptors, as $PGF_{2\alpha}$ was found to enhance OT receptor binding affinity in term myometrial samples (Baxi et al., 1980; Fukai et al., 1984).

OBE002 and atosiban treatments together had only an additive inhibitory effect on OT-induced contractions compared with atosiban alone. Similar to OT receptor signaling, the binding of $PGF_{2\alpha}$ to its receptor triggers G protein–mediated signal transduction pathways, leading to the increase in the levels of intracellular Ca^{2+} and subsequent myometrial contractions. It has been well established that a variety of G protein–coupled receptors can form both homo- and heterodimers which can affect their downstream signaling (Jonas and Hanyaloglu, 2017). Therefore, we postulate that cross-talk between FP receptors and oxytocin receptors may occur via receptor dimerization (or multimerization) or via the shared signaling pathways leading to contractions. Confirming receptor dimerization and deciphering the mechanisms involved in the cross-talk of FP and OT receptors represent part of our future work. In combination with OBE002, the calcium channel blocker nifedipine showed a synergistic effect on suppressing OT-induced contractions. Using different signaling pathways, both drugs may enhance each other's effects without competition.

In a model of spontaneous uterine activity in late-term anesthetized pregnant rats, intravenous or oral OBE022 and OBE002 were able to reduce spontaneous uterine contractions in a dose-dependent manner.

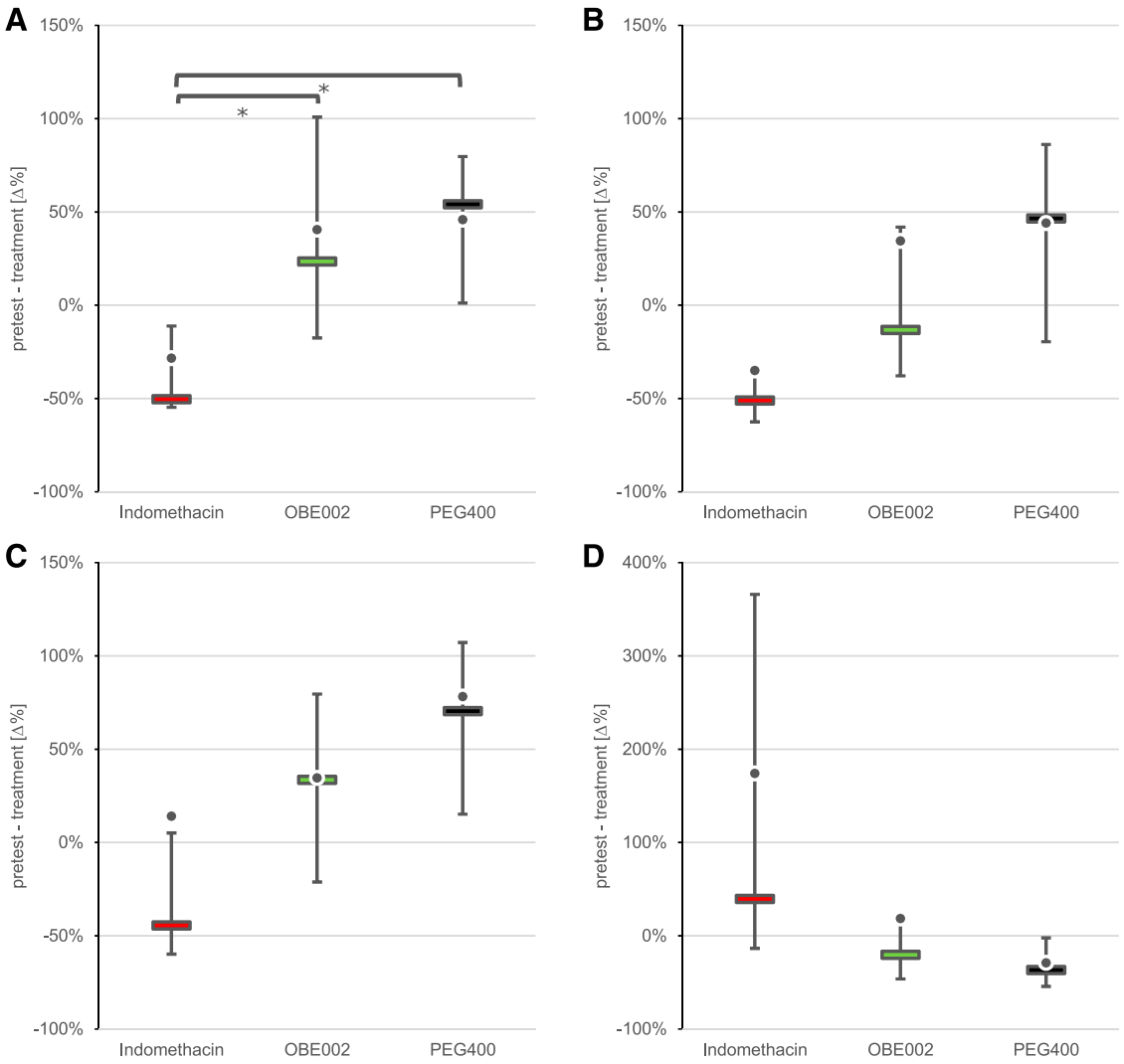

Fig. 8. Percentage differences (pre- vs. post-treatment) of renal function parameters of newborn rabbits treated with OBE002 (n = 13), indomethacin (n = 13), or vehicle (PEG400) (n = 9). (A) UFR. (B) GFR. (C) RBF. (D) RVR. Values are medians (−) ± interquartile range; means are represented by •. *$P < 0.05$

In the pregnant mice model, the antiprogestin RU486 induced preterm birth by blocking progesterone action and triggering the endocrine changes seen in labor (Garfield et al., 1987; Cirillo et al., 2007). Blocking FP activation with OBE022 caused delayed parturition. This may have occurred through inhibition of the action of $PGF_{2\alpha}$ in causing regression of the corpus luteum, essential for the normal withdrawal of progesterone in the mouse at the time of labor, through blocking FP in the myometrium, or through a combination of both. Gene knockout studies of the FP receptor showed that parturition was impaired, and that the failure of onset was primarily due to the absence of prepartum fall in circulating progesterone, which is required for uterine activation and contractile activity in rodents (Sugimoto et al., 1997). However, in addition to the luteolytic function of FP, our findings clearly demonstrate a myometrial effect and inhibition of uterotonic pathways in isolated human uterine smooth muscle and in the pregnant rat model of uterine contractility. Uterine activity was not fully abolished by treatment with OBE022. This would be expected since other factors, including other prostaglandins and oxytocin, mediate contractions at term. Indeed, the combination of OBE022 and the calcium channel blocker nifedipine was more effective than OBE022 alone. Importantly, treatment with OBE022 or nifedipine alone or combined had no effect on pup survival postdelivery, which was close to 100% in all RU486-treated mice. Nifedipine, used off label, is a common tocolytic used in the management of acute preterm labor. These data establish a rationale for targeting the FP receptor in combination with nifedipine as an optimized strategy for preventing or delaying preterm birth. Although FP and EP receptor expressions may be different in the human, rat, and mouse uterus, there is strong evidence that all are expressed and functional in these species (Myatt and Lye, 2004; Olson, 2005; Grigsby et al., 2006; Olson and Ammann, 2007). Having used controlled models previously tested with FP receptor antagonists, we believe that different receptor expression across species would not have altered the interpretation of our results.

To ensure the absence of fetal adverse effects observed with indomethacin, the most commonly used NSAID tocolytic, we performed OBE022/OBE002 safety pharmacology studies on kidney function, patency of the fetal DA, and platelet

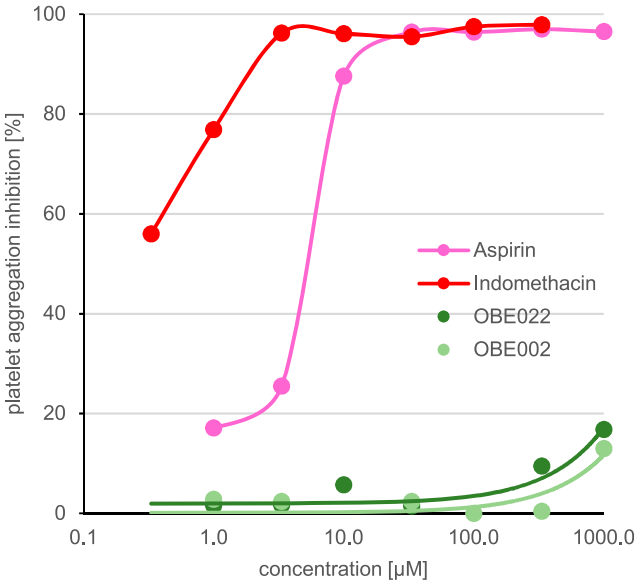

Fig. 9. Effects of OBE002, OBE022, and NSAIDs on human platelet aggregation induced in vitro by 1 mM arachidonic acid.

aggregation. The inclusion of appropriate positive and negative controls as well as OBE022/OBE002 concentrations markedly above clinical exposure levels ensured that species differences in receptor expression would not impact the interpretation of results.

Adverse impairment of renal function has been described in human neonates born to mothers treated with indomethacin to prevent a premature onset of labor (van der Heijden et al., 1988; Pomeranz et al., 1996). The significant impairment of the fetal urine production rate, GFR, or free-water clearance was acute and usually transient, but several cases of severe and sometimes irreversible renal insufficiency were reported (van der Heijden et al., 1988; Norton, 1997; Peruzzi et al., 1999; Sawdy et al., 2003).

We used the well described newborn rabbit model for the developing kidneys of premature infants and compared the renal hemodynamic effects of OBE002 and indomethacin (Guignard, 2002). As expected, indomethacin induced intense vasoconstriction and led to changes in UFR, GFR, and RBF (Duarte-Silva et al., 1986). There were no indomethacin-like renal effects in animals treated with OBE002. One of the limitations of our study was the level of interindividual variability of test parameters, which is inherent to this model. In line with other authors, we used the individual percentage changes of renal parameters before and after drug administration for effect evaluation. We also applied the same parameter calculation formulae and correction factors which led to FF values outside expected biologic ranges (i.e., FF $>$ 100%) in single animals. Thus, we repeated the analyses excluding the data of these animals and obtained similar results. PGE_2 and prostacyclin appear to be the best-defined and most important prostaglandins for renal function. These vasodilatory prostaglandins increase renal blood flow and glomerular filtration rate under conditions associated with decreased actual or effective circulating volume (Kim et al., 2008). Indomethacin blocks the synthesis of these prostaglandins. This may explain the impairing effect of indomethacin on renal function. In contrast, OBE002 is an antagonist of the FP receptor with high selectivity against all other prostaglandin receptors. The FP receptor is expressed in the distal convoluted tubule, a specific region along the nephron. However, the role of the $PGF_{2\alpha}$/FP system, if any, in regulating renal function is poorly defined. The selective FP agonist fluprostenol has no renal effects, which, taken together with our data, suggests that $PGF_{2\alpha}$ does not have important renal functions (Breyer and Breyer, 2000).

In humans, the patency of the fetal DA is maintained by PG produced locally and in the placenta. A decrease in DA patency leading to right ventricular collapse would severely compromise the fetus. Studies in mice, rats, and humans have demonstrated vasoconstriction of the fetal DA after administration of NSAIDs (Reese et al., 2006; Toyoshima et al., 2006; Yokoyama et al., 2014). In utero, constriction of the DA can lead to tricuspid regurgitation, irreversible pulmonary hypertension, and fetal death. In neonates, antenatal indomethacin treatment has been associated with pulmonary hypertension and patent DA (Norton, 1997; Smith, 1998; Loudon et al., 2003).

We used the rat model of DA constriction described by Toyoshima et al. (2006) to confirm the difference in the effect of OBE002 compared with indomethacin. Despite a limited number of animals used in this study, we had robust data to show no OBE002-related DA constriction, whereas indomethacin caused constriction of the fetal rat DA in line with previously published data (Reese et al., 2006; Toyoshima et al., 2006).

Critical roles for PGE_2 and the EP_4 receptor subtype have been established in maintaining both the patency of the DA vessel in utero and its closure at birth via EP_4 signaling (Segi et al., 1998; Gruzdev et al., 2012; Yokoyama et al., 2014). PGE_2 levels are high during the late phase of pregnancy and drop precipitously at birth. Levels of PGE_2 would also rapidly decrease in response to maternal NSAID treatment, thus causing fetal DA constriction. Our data suggest that $PGF_{2\alpha}$ does not play a major role in the patency of the DA, which is an important observation for the future clinical safety of OBE022 as a candidate drug for preterm birth.

Inhibition of platelet aggregation may be an aggravating factor for IVH, a serious adverse effect observed in fetuses and neonates of preterm labor mothers treated with NSAIDs (Hammer et al., 2015). In our ex vivo model, OBE022 and OBE002 did not inhibit human platelet aggregation, whereas significant inhibition of platelet aggregation was observed for indomethacin and aspirin. Thus, OBE022 and OBE002 confirm selectivity for FP and may not aggravate IVH.

In conclusion, we have developed the prodrug OBE022 as an orally available, highly selective, potent tocolytic agent. The parent drug and main circulating species OBE002 inhibited spontaneous and OT- and $PGF_{2\alpha}$-induced human myometrial tissue contractions alone and more effectively in combination with atosiban or nifedipine. Both OBE022 and OBE002 reduced spontaneous contractions in pregnant rats. In pregnant mice, oral OBE022 delayed RU486-induced parturition. OBE022 in combination with nifedipine exerted synergistic effects on the delay of delivery. Thus, OBE022 in combination with existing tocolytics may be a more effective strategy for preventing or delaying preterm delivery.

In contrast to indomethacin, OBE022 does not alter renal dynamics in newborn rabbits, does not close the DA in fetal

rats, and does not inhibit human platelet aggregation. OBE022 may hence represent a safe clinical candidate for preterm labor treatment—clinical studies in preterm labor patients are ongoing (NCT03369262).

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Authorship Contributions

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Conducted experiments: Kim, Riaposova, Spézia, Gervais, Guillaume, Lluel, Méen, Lemaux, Terzidou.

Contributed new reagents or analytic tools: Pohl, Chollet, Gotteland.

Performed data analysis: Pohl, Chollet, Kim, Riaposova, Spézia, Gervais, Guillaume, Lluel, Méen, Lemaux, Terzidou, Bennett, Gotteland.

Wrote or contributed to the writing of the manuscript: Pohl, Chollet, Kim, Riaposova, Gervais, Lemaux, Terzidou, Bennett, Gotteland.

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