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SSR126768A (4-Chloro-3-[(3*R*)-(+)-5-chloro-1-(2,4-dimethoxybenzyl)-3-methyl-2-oxo-2,3-dihydro-1*H*-indol-3-yl]-*N*-ethyl-*N*-(3-pyridylmethyl)-benzamide, Hydrochloride): A New Selective and Orally Active Oxytocin Receptor Antagonist for the Prevention of Preterm Labor

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ABSTRACT

4-Chloro-3-[(3*R*)-(+)-5-chloro-1-(2,4-dimethoxybenzyl)-3-methyl-2-oxo-2,3-dihydro-1*H*-indol-3-yl]-*N*-ethyl-*N*-(3-pyridylmethyl)-benzamide, hydrochloride (SSR126768A), a new potent and selective, orally active oxytocin (OT) receptor antagonist was characterized in several biochemical and pharmacological models. In binding studies, SSR126768A showed nanomolar affinity for rat and human recombinant and native OT receptors (K_i = 0.44 nM) and exhibited much lower affinity for V_{1a}, V_{1b}, and V_2 receptors. In addition, it did not interact with a large number of other receptors, enzymes, and ion channels (1 μM). In autoradiographic experiments performed on at-term human pregnant uterus sections, SSR126768A dose dependently displaced [I^{125}]d$(CH_2)_5$[Tyr(Me)$_2$, Thr^4, Orn^8 ^{125}I-Tyr-NH_2^9]VT in situ labeling to OT receptors highly expressed in these tissues. In functional studies, SSR126768A behaved as a full antagonist and potently antagonized OT-induced intracellular Ca^{2+} increase (K_i = 0.50 nM) and prostaglandin release (K_i = 0.45 nM) in human uterine smooth muscle cells. In rat isolated myometrium, OT-induced uterine contractions were competitively antagonized by SSR126768A (pA_2 = 8.47). Similarly, in human pregnant myometrial strips, SSR126768A inhibited the contractile uterine response to OT. In conscious telemetrated rats, oral administration of SSR126768A (1–10 mg/kg) produced a competitive inhibition of the dose response to OT on uterine contractions up to 24 h at 3 mg/kg p.o.; no tachyphylaxis was observed after 4-day repeated treatment. Finally, SSR126768A (30 mg/kg p.o.) significantly delayed parturition in pregnant rats in labor similar to ritodrine (10 mg/kg p.o.). Thus, SSR126768A is a potent, highly selective, orally active OT receptor antagonist with a long duration of action. This molecule could find therapeutic application as a tocolytic agent for acute and chronic oral management of preterm labor.

Preterm labor (at less than 37 completed weeks of gestation) occurs in approximatively 10% of births, accounts for 70% of all neonatal mortality and morbidity, and represents one of the highest costs per patient in health care budgets. Even though survival of preterm infants has increased due to the development of neonatal intensive care units, there has been no decrease in the rate of premature birth in the past 30

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ABBREVIATIONS: OT, oxytocin; AVP, arginine vasopressin; OTR, oxytocin receptor; SSR126768A, 4-chloro-3-[(3*R*)-(+)-5-chloro-1-(2,4-dimethoxybenzyl)-3-methyl-2-oxo-2,3-dihydro-1*H*-indol-3-yl]-*N*-ethyl-*N*-(3-pyridylmethyl)-benzamide, hydrochloride; SSR149515, ((2*S*,4*R*)-1-[5-chloro-1-[(2,4-dimethoxyphenyl)-sulfonyl]-3-(2-methoxyphenyl)-2-oxo-2,3-dihydro-1*H*-indol-3-yl]-4-hydroxy-*N*,*N*-dimethyl-2-pyrrolidine carboxamide, isomer(−)); SR49059, ((2*S*)-1-[(2*R*,3*S*)-(5-chloro-3-(2-chlorophenyl)-1-(3,4-dimethoxybenzenesulfonyl)-3-hydroxy-2,3-dihydro-1*H*-indole-2-carbonyl]-pyrrolidine-2-carboxamide); SR121463A, (1-[4-(*N*-*tert*-butylcarbamoyl)-2-methoxybenzene sulfonyl]-5-ethoxy-3-spiro-[4-(2-morpholinoethoxy)cyclohexane]indol-2-one, fumarate, equatorial isomer; PG, prostaglandin; AM, acetoxymethyl ester; UtSMC, uterine smooth muscle cell; [^{125}I]OVTA, d$(CH_2)_5$[Tyr(Me)$_2$, Thr^4, Orn^8 ^{125}I-Tyr-NH_2^9]VT; CHO, Chinese hamster ovary; $[Ca^{2+}]_i$, intracellular Ca^{2+}; ANOVA, analysis of variance; IUP, intrauterine pressure.

years. Importantly, it has been shown that neonatal survival improves significantly with gestational age and is closely related to the fetal weight (McLean et al., 1993, 1995; Hall et al., 1997). Therefore, the aim of preterm labor treatments is to maintain uterine quiescence using tocolytics (i.e., uterine-relaxing compounds) so as to increase the gestational age of delivery, to improve neonatal outcome and thus diminish both mortality and morbidity. Preterm labor drug development has been hampered by the lack of understanding of the initiating mechanisms of labor, marked species differences in the hormonal control of parturition, ethical considerations making it difficult to perform clinical trials during human pregnancy, and finally, the absence of clinical and biochemical tests for a definitive diagnosis of preterm labor (Smith et al., 2002). Thus, tocolytic agents include several classes of nonlicensed drugs in this indication such as magnesium sulfate, calcium channel blockers, nitric oxide donors, and prostaglandin synthase inhibitors. Atosiban, an OT/AVP peptide receptor antagonist, recently launched in Europe, and β_2-adrenergic agonists, such as Ritodrine, are the only licensed tocolytic agents. However, the former is a peptide molecule to be administered parenterally and is a more potent V_{1a} than OT receptor antagonist in humans, and the latter has been associated with severe maternal and fetal (mainly cardiovascular) adverse effects and tachyphylaxis (Goodwin and Zograbyan, 1998; Vatish and Thornton, 2002; Schwarz and Page, 2003).

There is a strong rationale for the design of pure OT receptor antagonists for preterm labor. Oxytocin is the most potent of the endogenous stimulants of uterine contractions and several lines of evidence support a key role for the OT/OTR system in the regulation of parturition and in preterm labor. Before the onset of labor, OT concentrations and uterine sensitivity to OT markedly increase, concomitant with a strong up-regulation of OT receptors in the myometrium where OT further stimulates the release of $PGF_{2\alpha}$/PGE_2 actively involved in the process of parturition. An increase in OT levels and OT receptors associated with preterm labor has also been reported. Moreover, synthetic OT is commonly used to induce labor and peptide oxytocin antagonists have been shown to inhibit preterm uterine contractions (Mitchell and Schmid, 2001). To overcome the problem of poor oral bioavailability associated with peptide compounds such as Atosiban, small molecules (mainly piperidine and benzazepine derivatives), selective for OT receptors, have been synthesized by Merck as orally active OT receptor antagonists but have been discontinued for pharmacokinetics reasons (Bell et al., 1998). More recently, a pyrrolidine derivative has been reported to possess oral efficacy in anesthetized pregnant rats (Cirillo et al., 2003).

In the present study, we report on the biochemical and pharmacological characterization of 4-chloro-3-[(3*R*)-(+)-5-chloro-1-(2,4-dimethoxybenzyl)-3-methyl-2-oxo-2,3-dihydro-1*H*-indol-3-yl]-*N*-ethyl-*N*-(3-pyridylmethyl)-benzamide, hydrochloride (SSR126768A) (Fig. 1), the first member of an original chemical series of highly potent, selective, and orally active OT receptor antagonists. Special attention was paid to the evaluation of the general selectivity of this compound with regards to AVP receptors and in other biological systems. Because marked species differences exist for AVP/OT receptors in terms of binding affinity and pharmacological properties, SSR126768A was extensively studied in various animal and human preparations expressing recombinant and native OT receptors and on human pregnant uterus as well (Pettibone et al., 1992; Kawamata et al., 2003). Inasmuch as OT controls uterine contractions and parturition, the in vivo uterine activity of SSR126768A was studied in conscious rats using, for the first time, a telemetric recording system comparable with that developed in primates (Carbonne et al., 1998). Finally, the efficacy of this molecule on parturition delay was assessed in pregnant rats. We demonstrate that SSR126768A is a potent, selective, and orally effective OT receptor antagonist that does not induce tachyphylaxis after repeated treatment and delays parturition in pregnant rats. Thus, SSR126768A exhibits a promising therapeutic profile for the acute and chronic treatment of preterm labor.

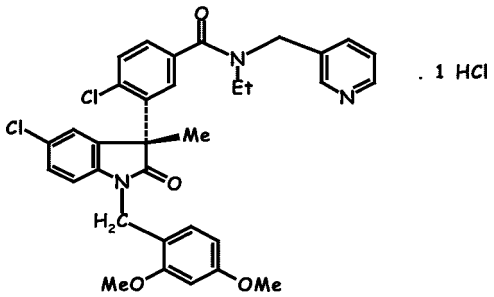

Fig. 1. Chemical structure of SSR126768A.

Materials and Methods

Materials

The nonpeptide molecules SSR126768A, SR49059, SSR149415, SR121463, and Atosiban were synthesized at Sanofi-Synthélabo Recherche (Toulouse, France). The chemical structures were determined by ^1H and ^{13}C NMR, mass spectrometry, and infrared spectroscopy. The purity, measured by high-pressure liquid chromatography, thin layer chromatography, and elemental analysis, was >98%. For in vitro binding experiments, the compound was initially dissolved in dimethyl sulfoxide at a concentration of 10^{-2} M and then diluted in the appropriate test solvent. In functional studies on human pregnant uterus, SSR126768A (0.25 mM) was dissolved in a mixture of absolute ethanol and distilled water [40:60 (v/v)] and then diluted in distilled water. The maximal final concentration of ethanol in the organ bath was 0.26%, and this concentration did not alter the rhythmic contractile activity. Doses were expressed as the nonsalified compounds. AVP, OT, and Pluronic F-127 were from Sigma-Aldrich (St. Louis, MO). Fura 2-acetoxymethyl ester (fura 2-AM) was from Molecular Probes (Eugene, OR). Bovine serum albumin fraction V was obtained from IBF (Paris, France). Uterine smooth muscle cells (UtSMC) and the corresponding cell culture medium were from Cambrex-BioWhittaker (Emerainville, France). All other cell culture reagents were from Roche Diagnostics (Meylan, France). Tris, $MgSO_4$, and dimethyl sulfoxide were purchased from Merck-Clevenot (Nogent sur Marne, France). All other chemicals were from Prolabo (Paris, France). The radioligands [^{3}H]AVP [8-L-arginine, [phenylalanyl-3,4,5–3*H*(N)]-vasopressin; 75 Ci/mmol], [^{3}H]SR121463 (47.5 Ci/mmol), and [^{125}I]OVTA [(d(CH_2)$_5$[Tyr(Me)$_2$, Thr^4, Orn^8 ^{125}I-Tyr-NH_2^{9}]VT; 2000 Ci/mmol] were synthesized by PerkinElmer Life Sciences (Boston, MA).

Biological Material

Female Sprague-Dawley CD rats (200–250 g) purchased from Charles River Laboratories (L'Arbresle, France) and female Wistar

rats (300 g) from CEJ Janvier (Le Geneste-Saint-Isle, France) were used for membrane preparations for binding studies and in vitro/in vivo uterine activity measurements. Female Sprague-Dawley (OFA; Oncins France souche A) pregnant rats were obtained from Iffa Credo (L'Arbresle, France). Male homozygous Brattleboro rats with central diabetes insipidus (300–350 g) were from Harlan (Indianapolis, IN). Water and chow were available ad libitum. All protocols performed have been approved by the Animal Care and Use Committee of Sanofi-Synthélabo Recherche. Myometrial biopsies were obtained from pregnant women with normal uncomplicated pregnancies who were delivered by elective caesarean section before the onset of labor (38–40th week of pregnancy) because of previously diagnosed cephalopelvic disproportion. Myometrial strips, excised from the longitudinal layer at the antiplacental site, were immediately placed in preoxygenated Krebs' solution at 4°C (118 mM NaCl, 5.4 mM KCl, 2.5 mM $CaCl_2$, 0.6 mM KH_2PO_4, 1,2 mM $MgSO_4$, 25 mM $NaHCO_3$, and 11.7 mM glucose). Tissue samples were dissected free from serosa and used immediately for functional studies or quickly frozen and stored at −80°C for autoradiographic experiments or membrane preparations for binding studies. This project was approved by the human subject ethical review committees of our institutions (Comité Consultatif de Protection des Personnes pour la Recherche Biomédicale, Dijon, France) and written informed consent was obtained from all donors.

In Vitro Experiments

Cell Culture and Membrane Preparation. Ltk$^-$ cells (murine fibroblasts) were transfected with the cDNA coding for the human OT receptor. CHO-DHFR-cells (DXB11) were transfected with an expression vector derived from plasmid 7055 containing the cDNA encoding the human V_2, V_{1a}, or V_{1b} receptor. Stably transformed cell lines were isolated as described previously (Serradeil-Le Gal et al., 1996, 2000). They were grown in 10 mM HEPES, pH 7.4, minimal essential medium supplemented with 5% fetal calf serum, 8 g/l sodium bicarbonate, and 300 μg/ml geneticin at 37°C in a humidified atmosphere containing 5% CO_2. Wild-type CHO cells were routinely grown in a similar culture medium. Human UtSMC (passages 4–10) were grown at 37°C in SmBM medium supplemented with human epidermal growth factor (0.5 μg/ml), human fibroblast growth factor (1 μg/ml), insulin (5 mg/ml), fetal calf serum (5%), and antibiotics. Culture medium was removed every other day, and cells were subcultured by treatment with 0.05% trypsin, 0.02% EDTA. Membranes from Ltk$^-$ cells, transfected with the human oxytocin receptors, UtSMC and CHO cells expressing the human V_{1a}, V_{1b}, and V_2 receptors, were prepared as described previously (Serradeil-Le Gal et al., 1996). Briefly, cells were harvested, washed twice in phosphate-buffered saline without Ca^{2+} and Mg^{2+}, Polytron-homogenized in lysis buffer (15 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, and 0.3 mM EDTA), and centrifuged at 100*g* for 5 min at 4°C. Pellets were washed in a buffer consisting of 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$ and centrifuged at 44,000*g* for 20 min at 4°C. Membranes were suspended in this buffer and protein contents were determined. Aliquots of membranes were used immediately or stored at −80°C. Rat kidney (papilla and inner medulla), liver, and mammary glands were prepared as described previously (Serradeil-Le Gal et al., 1993, 1996).

Binding Studies. Binding of [^{3}H]AVP to rat liver and to CHO membranes expressing the human V_{1a}, V_{1b}, or V_2 receptors were performed as described in Serradeil-Le Gal et al. (2002). Binding assays of [^{3}H]SR121463 to rat kidney medullary membranes were conducted according to Serradeil-Le Gal et al. (2000). Binding of [^{125}I]OVTA, a peptide OTR antagonist ligand, to rat nonpregnant uterus, Ltk$^-$ cell membranes expressing the human uterine oxytocin receptor, UtSMC, and human pregnant uterus were performed in an incubation medium (0.2 ml) containing 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM EGTA, 0.1% bovine serum albumin, 0.1% bacitracin, and ^{125}I-OT antagonist (0.02–3 nM for saturation experiments or 0.1 nM for competition studies), and increasing amounts of the compound to be tested (SSR126768A and reference peptides). The reaction was started by the addition of membranes (5–50 μg/assay) and incubated at equilibrium for 20 min at 25°C. Saturation binding experiments were performed in UtSMC or Ltk$^-$ membranes from cells transfected with the human OT receptor in the absence (control) or presence of SSR126768A (0.5, 1, and 2 nM). The reaction was stopped by adding 4 ml of ice-cold buffer followed by filtration through GF/C Whatman glass microfiber filters presoaked in ice-cold buffer. Filters were washed twice with 4 ml of ice-cold buffer and counted for radioactivity in a gamma counter (Amersham Biosciences AB, Uppsala, Sweden). Nonspecific binding was determined in the presence of 10 μM unlabeled OT.

Binding data analysis. The IC_{50} value was defined as the concentration of inhibitor required to obtain 50% inhibition of the specific binding. Inhibition constant (K_i) values were calculated from the IC_{50} values using the Cheng and Prusoff equation (Cheng and Prusoff, 1973). Data for equilibrium binding [apparent equilibrium dissociation constant (K_d), maximum binding density (B_{max})], and competition experiments [IC_{50}, Hill coefficient (n_H)] were analyzed using an iterative nonlinear regression program (Serradeil-Le Gal et al., 1993).

Autoradiography. Serial sections (16 μm in thickness) from frozen human pregnant uterus were mounted onto gelatin chrome-alum slides, rinsed to eliminate endogenous OT, and incubated with 0.05 nM [^{125}I]OVTA with (nonspecific binding) or without (total binding) 10 μM unlabeled OT. After 60 min. incubation at room temperature, the sections were washed three times for 10 min each in ice-cold binding buffer, dipped briefly in distilled water, and dried under a stream of cold air. Rinsed labeled sections were placed on a phosphor-imaging plate (Fuji, Tokyo, Japan) for 2 days and further analyzed with a Bio-Image analyser (BAS 2000; Fuji) as described previously (Serradeil-Le Gal et al., 2000).

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) Measurements. Subconfluent Ltk$^-$ cells expressing the human OTR and human UtSMC, cultured in 175-cm^2 flasks as described above, were collected by trypsinization (0.05% trypsin, 0.02% EDTA) and centrifuged (230*g*, 5 min). As described in Serradeil-Le Gal et al. (2002), the cells were suspended in culture medium at a final concentration of 5×10^6 cells/ml and then incubated with 5 μM fura 2-AM and 0.02% Pluronic F-127 at 30°C for 20 min under continuous shaking. At the end of the incubation, the cells were centrifugated (230*g*, 5 min) and washed with culture medium. The cells were washed twice in Hanks' buffer (137 mM NaCl, 5.4 mM KCl, 0.34 mM Na_2HPO_4, 5.5 mM glucose, 4.2 mM $NaHCO_3$, 0.8 mM $MgSO_4$, 10 mM HEPES-0.1 mM EGTA for the first wash only, pH 7.4). The cells were resuspended in this buffer to a final concentration of 2.7×10^6 cells/ml and kept at 4°C in the dark until use. Calcium transients were measured with an SLM 8000 C spectrofluorometer at 37°C (excitation at 340 and 380 nm, emission at 510 nm). Cytosolic free Ca^{2+} determination was performed as described by Grynkiewicz et al. (1985). Results were expressed as means ± S.E. and analyzed using RS1 software (BBN Domain, Cambridge, MA).

Prostaglandin Secretions in Human UtSMC. Human UtSMC were plated onto six-well plates at 5×10^4 cells/well and were grown for 8 days. The culture medium was then removed, and cells were maintained under quiescence for 2 days by adding the same culture deprived in fetal calf serum. To determine the dose response to OT, cells were preincubated with successive dilutions from 10^{-9} to 10^{-6} M. For the antagonism study, the compound to be tested was administered 5 min before the addition of $3 \cdot 10^{-9}$ M OT. The plates were further incubated at 37°C (5% CO_2, 80% humidity) for 18 h before the addition of 50 μM arachidonic acid. After 15 min, the medium was removed and frozen at −80°C. PGE_2 and $PGF_{2\alpha}$ were measured on the frozen supernatant by enzyme immunoassay using the PGE_2 and $PGF_{2\alpha}$ kit provided by Cayman Chemical (Ann Arbor, MI) The titration was performed in triplicate. Data are expressed as the mean ± S.D. and analyzed using RS1 software (BBN Domain).

Functional Studies in Human Pregnant Myometrial Strips. Myometrial tissues were cut into strips (8–10 mm in length by 2–3 mm in cross section) and suspended isometrically under a resting tension of 2 g in a 10-ml organ bath containing Krebs' solution (composition as described above) at 37°C and continuously gassed with a mixture of 95% oxygen and 5% carbon dioxide (pH 7.4) as described previously (Bardou et al., 2000). One end of each strip was connected to a force-displacement transducer, and tension changes were measured with Pioden strain gauges (UF1), amplified (EMKA, Paris, France) and recorded on a pen-writing oscillograph (L65514; Linseis, Selb, Germany). After 1 h during which the myometrial strips were washed every 15 min and the resting tension readjusted to 2 g, the strips were allowed to equilibrate for a further 1 h until they showed regular spontaneous rhythmic contractile activity. In a first series of experiments, once contractions became regular in amplitude and frequency, control concentration-response curves for oxytocin (10^{-10}–10^{-8} M) were determined by cumulative addition of oxytocin every 45 min. After washing and return to resting tone, SSR126768A (10^{-8}–10^{-6} M) was added to the bath, and 1 h later a second cumulative concentration-response curve for oxytocin (10^{-10}–10^{-7} M) was constructed. In a second series of experiments, after a resting period to obtain regular rhythmic contractile activity, the effect of SSR126768A on spontaneous activity was determined by cumulative addition of SSR126768A (10^{-7} and 10^{-6} M) added to the bath every 45 min. Finally, in a third series of experiments, to determine the lack of unspecific spasmolytic effect, SSR126768A was tested against contractile activity induced by KCl and norepinephrine. After equilibration and regular contractive activity, KCl (25 mM) or norepinephrine (10^{-7} M) was added to the bath for 30 min before or 1 h after SSR126768A (10^{-6} M). For all experiments, only one concentration of SSR126768A or vehicle was tested in each strip.

Analysis of results. In functional experiments, the effect of each agonist (OT, KCl, and norepinephrine) was expressed as a percentage of the initial amplitude or frequency of spontaneous contractions before or after SSR126768A. Data were expressed as means ± S.E. Differences among groups were determined by analysis of variance (ANOVA) and by Student's *t* test for paired data). The difference was considered significant when $p < 0.05$.

Organ Bath Experiments with Rat Myometrial Strips. The procedure was essentially the same as described by El Alj et al. (1993). Briefly, mature nulliparous Wistar female rats weighing approximately 300 g were caged individually at 22°C. Animals were ovariectomized and treated with 50 μg of estradiol benzoate as described in telemetric experiments. Rats were killed between 3 and 4 days after steroid treatment in the morning at 9:00 AM. Strips 15 × 3 mm (approximately 80 mg) were cut along the longitudinal axis and placed in organ baths (10-ml volume) to be processed in groups of eight. Isometric contractions were measured using BioScience UF1 tension transducers and recorded on a Pentium-PC and Gould recorder. The experiments were carried out at 32°C in a physiological salt solution gassed with 95% O_2, 5% CO_2, containing 144 mM NaCl, 6 mM KCl, 2.5 mM $CaCl_2$, 0.5 mM Mg_2SO_4, 30 mM $NaHCO_3$, 1 mM NaH_2PO_4, and 3 mM glucose, pH 7.4. Each experiment was preceded by a 1-h equilibration, during which each strip was washed three times and stretched to a baseline tension of 0.5 g.

OT was added cumulatively (0.001–1000 nM) to the bath every 3 min until the maximum response was attained. The response parameter used in all analyses of contractile responses was the integral under the tension-time curve for 3 min after OT was added. Agonist concentration response curves were fitted using GraphPad Prism (GraphPad Software Inc., San Diego, CA) to the equation $EA = (E_{max} \times Cn/[Cn + EC_{50}n]) + E0$, in which EA and C are the pharmacological effect and the concentration of the agonist, respectively. E0 is the starting basal activity, E_{max} is the asymptote, EC_{50} is the concentration resulting in a response at $E_{max}/2$ and n is the slope (pseudoHill coefficient). To account for possible variations in cross-sectional areas of strips, the E_{max} values of contractile agonists were simply divided by tissue wet weight (grams) of the sample. An estimation of the pA_2 values (Arunlakshana and Schild, 1959) for the OTR antagonist against OT (OT concentration range 3.16–100 nM) was determined by incubating the tissue with 1 or 100 nM of the antagonist that was allowed to equilibrate for 15 min before addition of OT for the construction of the concentration response curve.

In Vivo Experiments

Telemetric Recording of Rat Uterine Activity in Conscious Rats. *Surgical implantation of the telemetric transmitter.* Mature nulliparous Wistar female rats weighing approximately 300 g (CERJ, Le Genest-Saint-Isle, France) and caged individually at 22°C, were used. Intrauterine pressure (IUP) changes were monitored using an implantable telemetric transmitter equipped with a fluid-filled sensor catheter (type TA11PA-C40; Data Sciences International, St. Paul, MN). Surgery was carried out under general isoflurane anesthesia (2% in 0.4 l min^{-1} of oxygen) and aseptic conditions to prevent any postoperative infections. The uterine horns and ovaries were exposed by mid-ventral laparotomy. Bilateral ovariectomy was made and terminated by suturing the ovarian ends of both uterine horns with a silk suture. A trans-uterine puncture was made to insert the tip of the pressure-sensitive catheter (0.7 mm in diameter) into the lumen of the left uterine horn, toward the cervix. The uterine puncture was closed and the catheter was glued into place to the uterine wall with a small drop of tissue adhesive (Histoacryl; Braun, Melsungen, Germany). The outer portion of the catheter was fixed to the uterine horn with a silk suture. The body of the telemetric transmitter was positioned into the animal flank and attached with three silk sutures to the inner abdominal wall. The abdomen was closed in two layers. Standard postoperative care consisted in placing the animal in its cage on a heating carpet until full recovery from anesthesia.

Animals were kept under 12:12 (L:D) illumination and had free access to standard rat pellets and water ad libitum. They were given, intramuscularly, 50 μg of estradiol benzoate in oil to ensure a permanent estrus-like hormonal condition. Usually, a clear IUP signal could be recorded safely during 15 to 21 consecutive days on a same animal. After a 5-day recovery period, the implanted rats were brought to the recording room and placed unrestrained in their individual cages with free access to food and water. A telemetric receiver (type RMC-1; Data Sciences International) was placed under the cage and connected by a radioanalogic signal adapter (type R11-CPA; Data Sciences International) to a Pentium PC 400 Hz equipped with a PCI-MOI-16E digitizing hardware card and BioBench software (National Instruments, Austin, TX). The IUP signal was digitized at 10 Hz. For quantitative analysis, uterine activity was assessed by calculating the integral of the IUP curve, using BioBench software, over a 5-min period before OT administration (control period), and every 5 min after intramuscular administration of cumulative doses of OT. The results were expressed as the percentage of control uterine activity.

Product administration and construction of OT dose-response curves. OTR antagonists at various concentrations (final concentration to animals 1, 3, and 10 mg/kg) were suspended in 0.6% methylcellulose in water. Oral gavage (1 ml) of the methylcellulose solution alone or of the methylcellulose + OTR antagonist was administered to rats 1, 5, or 24 h before the construction of OT dose-response curves.

Cumulative doses of OT in saline 0.9% were administered intramuscularly (0.2 ml) every 5 min. Final estimated plasma concentrations (0.01–1000 nM, by increments of 1 log units) were calculated on the conventional basis of a volume of distribution for the peptide of 67 ml/kg of animal wet weight. Agonist concentration response curves were fitted using GraphPad Prism to the equation $EA = (E_{max} \times C/[C + EC_{50}]) + E0$, in which EA and C are the pharmacological effect and the concentration of the agonist, respectively. E0 is the starting basal activity, E_{max}, is the asymptote, EC_{50} is the concentration resulting in a response at $E_{max}/2$.

Data analysis and statistics. The data from dose-response curves

obtained in rat telemetric experiments or in organ bath experiments were analyzed with GraphPad Prism by one- or two-way ANOVA, wherever appropriate. When significant effects were found ($p < 0.05$), the Bonferroni test was used for comparisons with control values.

Parturition Delay Measurements in Late-Term Pregnant Rats. The ability of drug treatment to delay parturition was evaluated in late-term pregnant rats in labor by measuring the number of rat pups 90 min after the first pup delivery. Pregnant female Sprague-Dawley rats (Iffa Credo) were housed in individual cages and were observed continuously from day 21 of gestation until complete delivery. Immediately on delivery of the first pup, animals were at once administered either with tocolytics (SSR126768A 10 and 30 mg/kg p.o. or Ritodrine 10 mg/kg p.o.) or vehicle (5% ethanol, 5% Tween 80 in distilled water for SSR126768A and distilled water for ritodrine), under a volume of 5 ml/kg. The number of rat pups observed 1 h 30 after delivery of the first pup was recorded for each animal ($n = 8$ animals/group). Data were expressed as the mean ± S.E.M., and the percentage of births versus the corresponding vehicle group was calculated.

Statistical analysis. Data were analyzed using parametric tests (Student's *t* test or one-way analysis of variance followed by Dunnett's test). Differences with a probability of error <5% were considered significant.

Results

Affinity of SSR126768A for Rat and Human OT Receptors and Selectivity Profile

In various preparations, extensively characterized using reference peptide and nonpeptide AVP/OT ligands, SSR126768A displays high nanomolar affinity for rat OTR and human recombinant (Ltk^-) or native OTR in uterine smooth muscle cells and pregnant uterus membranes (Table 1). As illustrated in Fig. 2A, SSR126768A dose dependently antagonized [^{125}I]OVTA binding to human uterine OTR with an affinity even higher than that of the natural hormone, OT (K_i values of 0.9 ± 0.2 and 6 ± 3 nM, respectively), whereas the peptide antagonist Atosiban exhibited much lower affinity for human OTR (K_i values of 561 ± 167 nM). Of note, this compound displayed higher affinity for human, and not for rat, V_{1a}R (Table 1) receptors in agreement with marked species differences already reported for this molecule, indicating that Atosiban is a more potent OTR antagonist in animal species and a potent V_{1a} receptor antagonist in human (Pettibone et al., 1992; Allen et al., 1997; Akerlund et al., 1999; Cirillo et al., 2003). Saturation binding experiments, performed in Ltk^- (data not shown) or uterine smooth muscle cells in the presence (0, 0.5, 1, and 2 nM) or absence of SSR126768A, indicate that SSR126768A is a competitive antagonist in inhibiting [^{125}I]OVTA binding. As shown on the Scatchard representation (Fig. 2B), the K_d was dose dependently decreased, whereas the B_{max} was not modified in agreement with a competitive profile. The K_i value calculated from Scatchard plots (0.44 ± 0.08 nM) was consistent with the K_i value obtained in competition studies (0.9 ± 0.2 nM) (Table 1).

The selectivity profile of SSR126768A was first assessed for OTR-related receptors (i.e., AVP V_{1a}, V_{1b}, and V_2 receptors) from rat and human origin. SSR126768A exhibited only a weak affinity for these receptors with an affinity about 100-fold lower at V_{1a} and V_{1b} human receptors and even much lower (>500) for human V_2R (Table 1). It is worth noting that SSR126768A discriminated between rat and human V_{1b} receptors, consistent with species variations reported for AVP/OT receptors (Table 1). To complete the functional selectivity profile of SSR126768A at AVP receptors, we studied this compound on Ca^{2+} transients in cells stably transfected with human V_{1a} or V_{1b} receptors. In both cell lines, SSR126768A (1 μM) was unable to increase intracellular Ca^{2+} when tested alone and decreased AVP-induced Ca^{2+} increase, showing an absence of agonist effect with an antagonist profile at higher concentrations. Similarly, SSR126768A was without effect on cAMP production on CHO cells expressing the human V_2 receptors and exhibited antagonist effects on AVP-induced cAMP production only at the highest concentrations (>10 μM) (data not shown). Finally, the selectivity of SSR126768A was assessed in several binding and enzyme biological assays in vitro ($n = 101$ tested). Tested at 1 μM, SSR126768A did not interact with receptors of nonpeptide (adenosine, adrenergic, angiotensin, benzodiazepine, cannabinoid, dopamine, histamine, acetylcholine, serotonine, progesterone, glucocorticoid, Ca^{2+}, Na^+ Cl^-, and K^+ channels) or peptide ligands (neuropeptide Y, endothelin, neurotensin, bradykinin, galanin, nociceptin, and somatostatin) nor with several enzymes (cycloxygenases, phosphodiesterases, angiotensin-converting enzyme, protein kinase C, monoamine oxidase, ATPase, acetylcholinesterase).

Autoradiography with SSR126768A in Human Pregnant Uterus Sections

Using [^{125}I]OVTA, a well-characterized OTR antagonist peptide ligand (Elands et al., 1987), OT (10 μM), and

TABLE 1

Comparative binding affinity of SSR126768A and Atosiban for human and rat oxytocin and vasopressin receptors

Binding assays were performed as described under *Materials and Methods* using CHO/LtK^- cells transfected with the corresponding AVP/OT receptor or native tissues constitutively expressing this receptor. The radioligand [^{125}I]OVTA was used in the different OTR binding studies and [^{3}H]AVP in V_{1a}, V_{1b}, and human V_2 receptor experiments. Binding assays on rat kidney medullary membranes were performed using [^{3}H]SR121463. Inhibition constants (K_i) were determined from competition experiments and calculated according to the Cheng and Prusoff (1973) equation. Values are the mean ± S.D. of data obtained from at least three independent determinations.

	K_i (nM)					
	OT			Rec. CHO		
Human	Rec Ltk Cells	UtSMC	Pregnant Uterus	V_{1a}	V_1b	V_2
SSR126768A	1.5 ± 0.8	0.9 ± 0.2	0.5 ± 0.2	143 ± 23		>1000
Atosiban	895 ± 238	561 ± 167	775 ± 238	4.7 ± 0.5	256 ± 69	3195 ± 728
Rat	Uterus			Liver	CHO	Kidney
SSR126768A	1.6 ± 0.3			99 ± 31	46 ± 3	>1000
Atosiban	215 ± 30			1059 ± 232	241	>1000

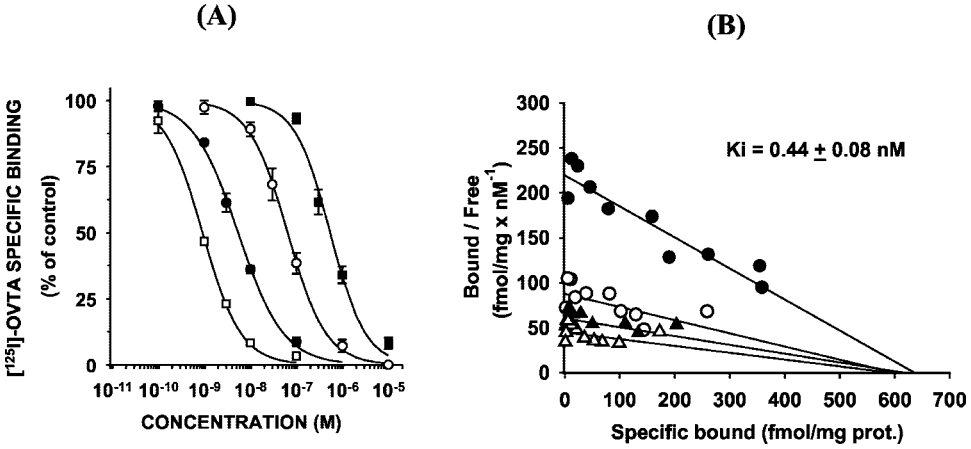

Fig. 2. Effect of SSR126768A on [^{125}I]OVTA binding to human OT receptors in UtSMC. A, inhibition of 0.1 nM [^{125}I]OVTA specific binding to human OT receptors by SSR126768A (□) and reference peptide compounds: AVP, ○; OT, ●; and Atosiban, ■. B, Scatchard plots of [^{125}I]OVTA binding to UtSMC membranes without (●) or with 0.5 nM (●), 1 nM (▲), and 2 nM (△) SSR126768A. Binding assays were performed for 20 min at 25°C in the presence of 30 μg/assay of UtSMC membranes as described under *Materials and Methods*. Results represent data from a typical experiment performed in duplicate, which was repeated three times without noticeable change.

SSR126768A, we studied for the first time the mapping and localization of OTR in human pregnant uterine samples by the autoradiographic technique. As illustrated in Fig. 3A, intense specific labeling was found in all the pregnant human myometrial strips studied excised from the longitudinal uterine layer ($n = 5$), showing high and constant expression of the OTR in the uterus at term; comparatively, nonpregnant human uterus samples studied under similar operating conditions exhibited much lower labeling (data not shown). SSR126768A (3.10^{-10}–10^{-7} M) inhibited [^{125}I]OVTA binding to its sites in a dose-dependent manner (Fig. 3B). Of note, at 100 nM, SSR126768A displaced more than 95% of [^{125}I]OVTA specific staining and quantitative analysis yielded a K_i value of 2.0 ± 0.4 nM ($n = 5$) in agreement with the affinity found for this compound at human OTR in membrane binding experiments (Table 1). SSR126768A thus represents a selective probe for studying in situ localization of OTR.

Functional Tests in Vitro

Effect of SSR126768A on OT-Induced Intracellular Ca^{2+} Increase. Activation of OTR has been associated with intracellular $[Ca^{2+}]_i$ elevation via a Gq protein-dependent pathway (Gimpl and Fahrenholz, 2001). To determine the agonist or antagonist profile of SSR126768A at OTR, we studied the effect of this compound on OT-induced $[Ca^{2+}]$ increase both in Ltk$^-$ cells transfected with the recombinant human OTR and in human UtSMC constitutively expressing the OTR. In these preparations, OT dose dependently increased $[Ca^{2+}]_i$ with an EC_{50} value of 13 ± 9 and 23 ± 13 nM, respectively. As shown in Fig. 4A, SSR126768A antagonized in a concentration-dependent manner 30 nM OT-evoked $[Ca^{2+}]_i$ elevation in UtSMC with a K_i value of 0.50 ± 0.28 nM ($n = 3$). Similar results were obtained in Ltk$^-$ cells expressing the recombinant human OTR ($K_i = 0.24 \pm 0.08$ nM) (data not shown). Interestingly, when tested alone up to 10^{-6} M, SSR126768A was devoid of agonist effects in inducing intracellular Ca^{2+} increase in these preparations.

Effect of SSR126768A on OT-Induced Prostaglandin Release in Human UtSMC. In the uterus, OT is a potent prostaglandin-releasing factor. $PGF_{2\alpha}$ and PGE_2 are key substances actively involved in the parturition process in animals and in humans (Mitchell and Schmid, 2001). The effect of SSR126768A was studied on OT-induced prostaglandin release using the model of cultured human UtSMC. In this preparation, an OT treatment dose dependently stimulated both $PGF_{2\alpha}$ and PGE_2 release with EC_{50} values of 9.2 ± 5.8 and 14 ± 10 nM ($n = 4$), respectively. As illustrated in Fig. 4B, SSR126768A totally antagonized OT (3 nM)-induced PGE_2 secretion in a dose-dependent manner with a K_i value of 0.45 ± 0.35 nM. Conversely, reference selective V_{1a} (SR49059), V_{1b} (SSR149415), and V_2 (SSR121463A) receptors antagonists (10^{-7} M) used to calibrate the model were devoid of significant effect on OT-evoked PGE_2 secretion, in agreement with a pure OTR-mediated effect (data not shown).

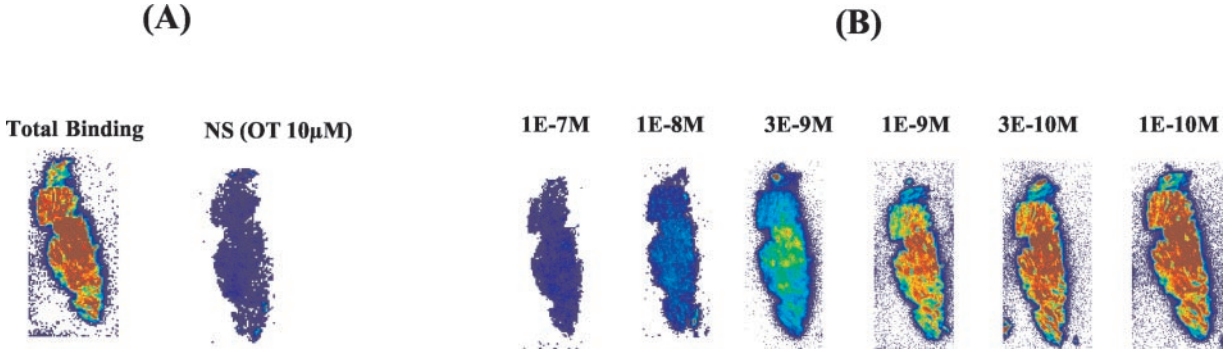

Fig. 3. Autoradiograms with [^{125}I]OVTA in human pregnant myometrium sections. A, binding of [^{125}I]OVTA in the absence (total binding) and in the presence of 10 μM OT (nonspecific binding, NS). B, autoradiography of [^{125}I]OVTA in the presence of increasing concentrations of SSR126768A (10^{-10}–10^{-7} M). Serial sections (16 μm in thickness) were incubated with 0.05 nM [^{125}I]OVTA as described under *Materials and Methods* and exposed onto a phosphor-imaging plate. The quantification of the images was performed with computer-assisted densitometry using a Bio-Image analyser as described in Serradeil-Le Gal et al. (2000). The red-brown color corresponds to the highest intensity of labeling. Results represent data from a typical experiment, which was repeated on five different preparations without noticeable change. Gradation color scale: lower to higher intensity of labeling.

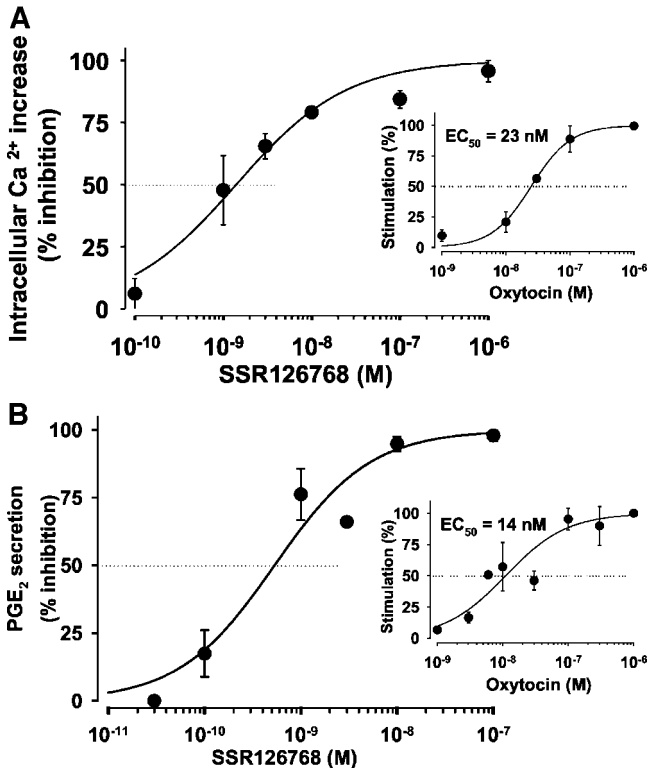

Fig. 4. Antagonism by SSR126768A of OT-induced $[Ca^{2+}]_i$ elevation (A) and PGE_2 release (B) in UtSMC in vitro. A, dispersed UtSMC cells ($4–5 \times 10^5$ cells/ml), preloaded with 2 μM fura 2-AM, were incubated with increasing concentrations of SSR126768A before stimulation with 30 nM OT as described under *Materials and Methods*. Inset, dose-response curve of OT. B, cultured UtSMC plated in six-well plates were stimulated with OT (3 nM) in the presence of increasing concentrations of SSR126768A. (10^{-10}–10^{-7} M). Eighteen hours after stimulation culture medium was removed and stored frozen at −20°C for later PGE_2 measurements by enzyme immunoassay. Data represent the mean (±S.D.) of data derived from three to five experiments.

Once again, no agonist OT-like effects on prostaglandin release were detected for SSR126768A when tested alone.

Effect of SSR126768A on OT-Induced Rat Myometrial Contractions in Vitro. As shown in Fig. 5, OT (0.1–1000 nM) caused a potent contractile effect in rat isolated longitudinal myometrial strips. SSR126768A (1 and 100 nM) produced a significant parallel rightward shifts in the OT concentration-response curve without significantly modifying the maximal contractile response, suggesting competitive antagonism. Schild plot of these data yielded an estimated pA_2 value of 8.47 with a slope not significantly different from 1 (0.88), suggesting potent and full competitive antagonism of SSR126768A at OT receptors.

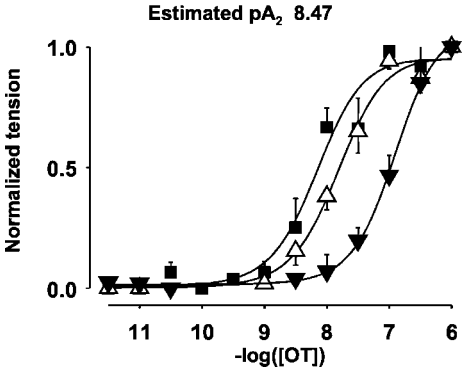

Fig. 5. Concentration-response curves of OT-induced contractions in rat isolated longitudinal myometrial strips in the absence (■) or presence of SSR126768A 1 nM (△) and 100 nM (▼). After 1-h equilibration, each strip was stretched to a baseline tension of 0.5 g (normalized tension); OT (0.001–1000 nM) was added cumulatively to the bath every 3 min. The estimated pA_2 value for SSR126768A against OT was determined by incubating the antagonist 15 min before addition of OT according to Arunlashana and Schild method. Results are the mean ± S.E.M. of five determinations.

In Vitro Activity of SSR126768A in Human Pregnant Myometrial Strips. Isolated human pregnant myometrial strips are highly sensitive to OT, which stimulates both contraction amplitude and frequency (EC_{50} of 1.5 ± 0.4 and 7.9 ± 2.2 nM, respectively; $n = 34$ strips out of 19 myometrium tested; data not shown). One-hour pretreatment of preparations with various concentrations of SSR126768A (10^{-8}–10^{-6} M) inhibited the response to OT in a concentration dependent manner; at 10^{-6} M an inhibition of about 60% was obtained on both parameters (Fig. 6, A and B). Schild analysis of these data yielded pA_2 values of 8.71 and 9.03 on frequency and tension parameters, respectively, with slopes not significantly different from 1, suggesting competitive antagonism by SSR126768A at OT receptors (Fig. 6, A and B). Additionally, SSR126768A exerted a significant effect on human myometrium spontaneous activity (20 and 40% inhibition at 10^{-7} and 10^{-6} M, respectively; data not shown). Of note, all the strips tested were highly sensitive to SSR126768A ($n = 8$ preparations). Interestingly, SSR126768A (10^{-6} M) was unable to antagonize the contractile response to KCl (25 mM) and norepinephrine (10^{-7} M), indicating a lack of unspecific spasmolytic effect, in agreement with the high specificity of this molecule (data not shown).

Effect of Oral Administration of SSR126768A on Uterine Activity Assessed by Telemetry in Conscious Rats. Uterine activity was measured in vivo by telemetric recording of the IUP in conscious rats. As shown on a typical recording in Fig. 7A, OT injected intramuscularly was a potent uterotonic peptide, which dose dependently stimulated uterine activity by increasing tension and frequency. On these recordings, SSR126768A (3 mg/kg p.o.), administered 5 h before the OT dose response, was a potent blocker of OT uterotonic effects. Oral administration of increasing doses of SSR126768A (1–10 mg/kg) shifted the dose-response curve for OT dose dependently to the right without significantly modifying the maximal contractile effect of OT, suggesting competitive antagonism as previously observed in rat uterus isolated preparations in vitro (Fig. 7B). Of note, the minimal effective dose was lower than 1 mg/kg p.o. at 5 h posttreatment ($p < 0.01$). In vivo repeated injections of OT at 1, 5, and 24 h post-treatment resulted in highly reproducible dose-response curves for OT on uterine contraction (data not shown) as indicated by similar EC_{50} values determined at the different times (Table 2). The time course of the effect of oral administration of SSR126768A (1–10 mg/kg) was recorded at 1, 5, and 24 h. As shown in Table 2, SSR126768A showed a rapid onset of action with a highly significant effect at 1 h after treatment ($p < 0.01$). SSR126768A exhibited a long duration of action because the dose of 3 mg/kg p.o. still induced significant antagonism on OT uterotonic response ($p < 0.05$) at 24 h. Moreover, when SR126768A was given repeatedly at 3 mg/kg for 4 days, the uterine relaxing effect of SSR126768A at 1 h was similar to that obtained after acute

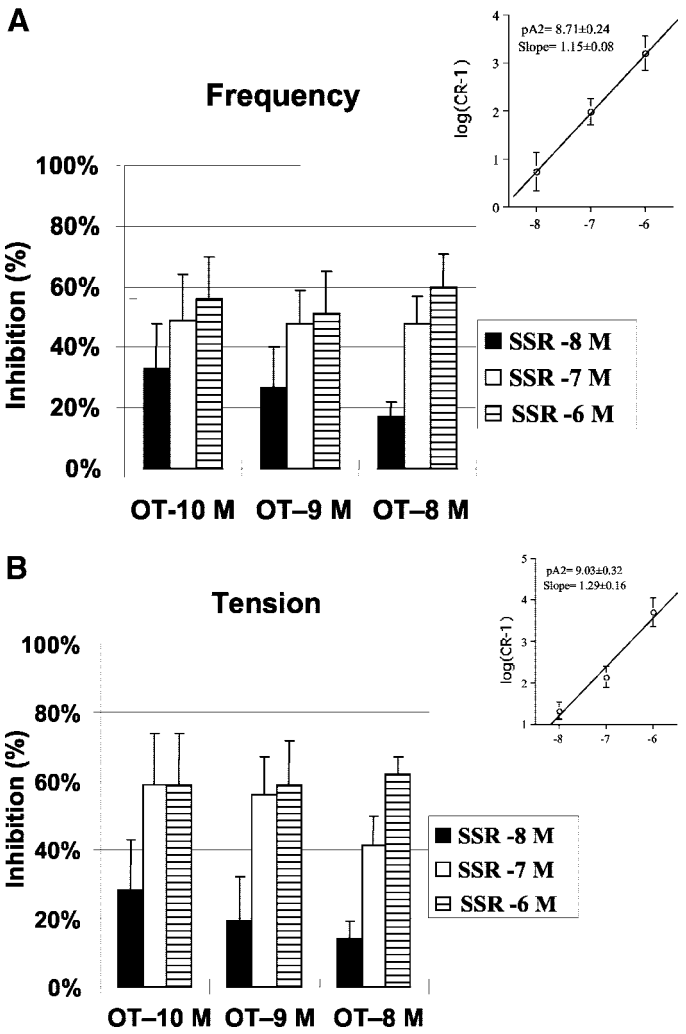

Fig. 6. Antagonism by SSR126768A of OT-induced increase in contraction rate (A) and tension (B) in human pregnant myometrial strips, in vitro. Myometrial strips, excised from the longitudinal layer, were immediately mounted in an oxygenated Krebs' solution and placed under 2-g resting tension for 1 h. SSR126768A [10^{-8} (■), 10^{-7} (□), or 10^{-6} (▤) M] or vehicle was added 1 h before the OT challenge (10^{-10}, 10^{-9}, or 10^{-8} M) as described under *Materials and Methods*. Inset, Schild analysis of data yielded pA_2 values of 8.71 and 9.03 on frequency and tension parameters, respectively, with slopes not significantly different from one. Data represented are the mean ± S.E.M. of seven to 15 determinations performed on five to eight independent preparations.

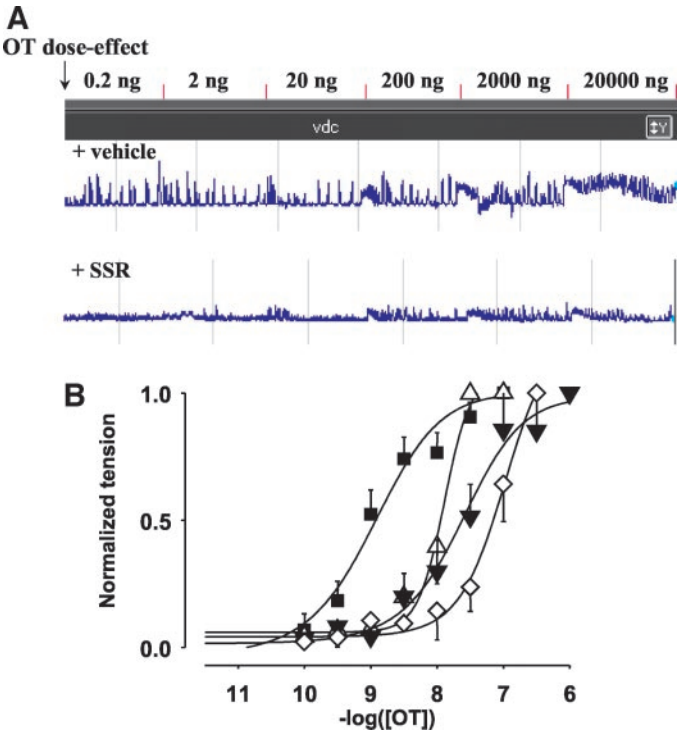

Fig. 7. Effect of oral administration of SSR126768A on OT-induced uterine contraction in conscious rats. A, typical recording from the cumulative dose response to OT in the absence (vehicle, i.e., 0.6% methylcellulose in water) or in the presence of SSR126768A (3 mg/kg) p.o. B, dose-response curve of OT-induced uterine contractions in vivo in the absence (■) or presence of SSR126768A 1 mg/kg (△), 3 mg/kg (▼), and 10 mg/kg (◇) at 5 h after an acute oral administration. IUP changes were monitored in female Wistar rats using a telemetric implant. The IUP signal was digitized at 10 Hz and for quantitative analysis the integral of the IUP curve was calculated over a 5-min period before (control period) and every 5 min after intramuscular cumulative doses of oxytocin administration. SSR126768A at various concentrations (1, 3, and 10 mg/kg p.o.) or vehicle alone (0.6% methylcellulose in water) was administered to rats 5 h before the OT dose-response curve. The results are expressed as the percentage of control uterine activity ± S.E.M.

TABLE 2

Time course of the effect of oral administration of SSR126768A (1, 3, and 10 mg/kg) on oxytocin-induced uterine contractions in conscious rats

SSR126768A (1, 3, and 10 mg/kg in 0.6% methylcellulose in water) was administered to rats 1, 5, and 24 h before OT administered intramuscularly every 5 min. Agonist concentration-response curves were fitted using the GraphPad Prism software (n = 3–8 animals/group). Statistical significance was assessed using a two-way ANOVA and the level of significance was taken as $p < 0.05$ for comparison with the corresponding OT control group.

OT EC_{50} (nM)				
OT Alone	OT + SSR126768A	Time (h)		
		1	5	24
	mg/kg			
0.78 [0.57–1.07]	1	27* [19–37]	17* [12–25]	
0.78 [0.57–1.07]	3	14* [11–19]	24* [16–34]	4.7* [2.5–8.8]
0.99 [0.64–1.53]	10	79* [58–110]	148* [98–224]	

treatment, showing absence of tachyphylaxis (data not shown). SSR126768A was devoid of agonist effects in stimulating uterine contractions when tested alone.

Effect of SSR126768A on Parturition Delay in Late-Term Pregnant Rats. Pregnant rats initiated labor from day 21 to day 22 of gestation. As shown in Fig. 8, treatment with Ritodrine (10 mg/kg p.o.) significantly decreased the litter size (50% inhibition) observed at 1 h 30 min compared with the vehicle group ($p < 0.05$). Similarly, SSR126768A (10 and 30 mg/kg p.o.) decreased in a dose-dependent manner the number of births recorded 1 h 30 min after treatment. The effect reached significance at 30 mg/kg p.o. ($p < 0.05$) with an inhibition of about 40% versus the vehicle group showing tocolytic properties for SSR126768A after oral administration. Interestingly, the compound seemed well tolerated, and no toxicity was observed at these doses.

Discussion

Even if the mechanisms that trigger the onset of labor are complex and multifactorial, ample evidence provides support for a major role played by the OT/OTR system in the initiation of labor in mammals and in the regulation of human

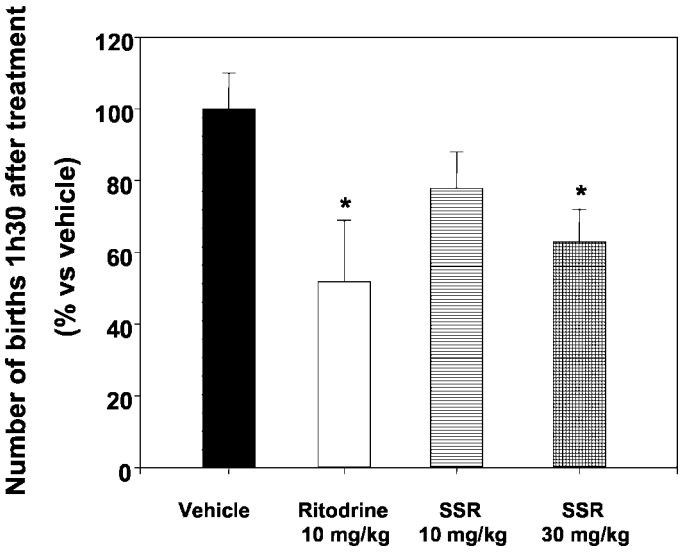

Fig. 8. Effect of oral treatment with SSR126768A and Ritodrine on parturition delay in termed pregnant conscious rats in labor. Late-term pregnant rats were observed from day 21 to 22 of pregnancy. Immediately after delivery of the first pup, animals were treated either with tocolytics [SSR126768A 10 mg/kg p.o. (▨) and 30 mg/kg p.o. (▩) or Ritodrine 10 mg/kg p.o. (□)] or vehicle (■). The number of rat pups measured 1 h 30 min after treatment was recorded for each animal (n = 8 animals/group). The pup number was measured 1 h 30 min after the first birth for each animal. Results are expressed as the percentage of births versus vehicle group. Data represent mean ± S.E.M. $p < 0.05$ (Dunnett's or Student's t test versus vehicle group).

parturition (Goodwin and Zograbyan, 1998; Mitchell and Schmid, 2001). It is still unclear how important this system is in preterm labor, but an increase in the OT/OTR tone has been associated with preterm labor (Fuchs et al., 1984). The relatively selective distribution of OTR in the uterus, the cell/tissue specific uterine up-regulation for OTR at the time of parturition, the paracrine/autocrine stimulation of the OTR in the uterus with large local fetal and maternal OT production, and the OT-stimulating effect on prostaglandin secretion support the development of pure OTR antagonists for the prolongation of gestation and might make such antagonists superior to currently used tocolytics in terms of both efficacy and safety. Several synthetic OTR antagonists have been designed by chemical modification of the natural hormone producing potent OT ligands with various selectivity, able, for some of them, to delay or to interrupt labor process when administered to pregnant animals (Chen et al., 1994; Demarest et al., 1989; Kobayashi et al., 1999; Manning et al., 2001) and to pregnant women as well, after intravenous infusion (Akerlund et al., 1987; Romero et al., 2002; The Worldwide Atosiban versus Beta-Agonists Study Group, 2003). In this context, nonpeptide OTR antagonists with good oral bioavailability are eagerly awaited for acute and chronic treatment of premature labor.

The present study describes the biochemical and pharmacological profile of SSR126768A, a highly potent and selective, orally effective OTR antagonist with marked tocolytic properties in vitro and in vivo. In binding experiments, nanomolar and subnanomolar potencies were obtained at recombinant and native OTR from UtSMC and pregnant uterus at term (K_i = 0.5–1.5 nM). Moreover, in situ autoradiographic studies performed on this latter tissue confirmed the strong interaction of SSR126768A with the human uterine OTR at term (K_i = 2 nM), highly expressed in this preparation. Indeed, plasticity of the OTR is a typical feature of this receptor, which is able to modify its binding properties and receptor number in the uterus. It has been shown that OTR density increases during pregnancy and before the onset of labor, uterine sensitivity to OT markedly increases as well (Mitchell and Schmid, 2001).

Because species differences are very common in the field of AVP/OT, it is worth noting that SSR126768A exhibits similar nanomolar affinity for rat and human OTR (Table 1) and for a wide number of animal species, including guinea pig, mice, and maccaca (data not shown). As a typical illustration of interspecies variations, the peptide antagonist Atosiban is a more potent V_{1a} receptor antagonist in human than in rats (Table 1) (Pettibone et al., 1992; Allen et al., 1997). Similarly, the nonpeptide compound, OPC21268, designed as a V_{1a}R antagonist in rats, turned out to be an OT-selective ligand for human OTR (Williams et al., 1995).

Because OT and AVP are closely related nonapeptides, the OTR shares structural similarities with the AVP receptor subtypes (V_{1a}, V_{1b}, and V_2 receptors), which can explain the difficulties in obtaining selective OTR molecules. For peptides, one of the most selective compounds for OTR is that with threonine in C4 position and ornithine in the C8 position (Chen et al., 1994; Manning et al., 2001). But, for nonpeptide molecules, no rule has been established, and the recently described pyrrolidine carboxamide derivative OTR antagonist exhibited very moderate OTR selectivity with only a 6-fold selectivity against V_{1a}R (Cirillo et al., 2003). Thus, the highly selective OTR profile of SSR126768A is an important characteristic of this molecule. First, in vitro, this compound has low affinity for human AVP receptors and the OTR selectivity versus these receptors is of about 100 for V_{1a}/V_{1b} and >600 for V_2 (Table 1); of note, this OTR selectivity is somewhat lower versus rat AVP receptors underlining some species variations. In functional studies on CHO cells expressing either V_{1a} or V_2 receptors, SSR126768A displayed antagonistic properties in antagonizing AVP-induced Ca^{2+} increase or cAMP accumulation, at higher concentration than those interacting with OTR. Second, we reported the general absence of interaction of SSR126768A (1 μM) with a large number (n = 101 bioassays tested) of AVP/OT unrelated receptors, ion channels, or enzymes and a lack of unspecific spasmolytic effect against KCl or norepinephrine contractions has been observed in functional human pregnant uterus in vitro. Thus, SSR126768A constitutes a helpful specific ligand for mapping animal and human OTR. One could also expect a particularly safe profile for this molecule judging from its specific targeted action at OTR.

Via OTR uterine activation, OT exerts a dual effect by directly contracting the myometrium and inducing prostaglandin secretion. Prostaglandins are crucial uterotonic substances acting synergically with OT on contraction; they play also a key role in the myometrial maturation by producing enzymes able to digest collagen, leading to cervical softening and ripening (Carbillon et al., 2001). Interestingly, in the human model of cultured UtSMC used in this study, OT-induced both Ca^{2+} increase, as an immediate cellular event of the contractile machinery, and PGE_2 release as a secondary step. We demonstrated that SSR126768A has powerful antagonist effects on both components with a subnanomolar potency (K_i = 0.50 and 0.45 nM, respectively) in agreement

with the affinity found for the OTR in these cells (Table 1). This cellular mechanism of action accounts for the strong inhibitory effect of SSR126768A on OT-induced uterine contraction in vitro and in vivo in rat and human models. In rat isolated nonpregnant uterus and in human termed pregnant myometrium, SSR126768A exerted pronounced inhibition against OT effects (pA_2 of about 9). Spontaneous contractions of human pregnant uterus were also sensitive to SSR126768A antagonism (20 and 40% inhibition at 10^{-7} and 10^{-6} M, respectively). Interestingly, the concentration of OTR has been shown to correlate with spontaneous uterine activity at term and constitutive activity of the OTR could explain this phenomenon, considered as an important factor for initiating labor (Wilson et al., 2001). Thus, SSR126768A may behaved as in inverse agonist at the OTR in human pregnant uterus strips in vitro, an additional property that can support the tocolytic effect of SSR126768A observed in vivo. However, a local production of OT cannot be excluded and could support, at least in part, the spontaneous activity observed in these preparations. Additionally, the partial efficacy of SSR126768A on spontaneous uterine contractions in vitro and on the progression of labor in rats in vivo, also suggests the involvement of other receptors and hormonal systems in controlling myometrial activity and parturition.

The potent effect of SSR126768A on exogenous OT-induced uterine contractions in conscious rats, after acute and repeated oral administration, and on the natural progression of labor in pregnant rats, demonstrates powerful tocolytic properties for this molecule in vivo together with excellent bioavailability. SSR126768A (1–10 mg/kg p.o.) showed a rapid onset of action, with an effect still significant 1 h after oral administration ($p < 0.01$), and a long duration of action because significant antagonism against OT uterine effect was still observed 24 h after treatment with a dose as low as 3 mg/kg p.o. Finally, we demonstrated that oral administration of SSR126768A caused a strong inhibition of parturition progression in rats in labor, similar at 30 mg/kg to that of ritodrine at 10 mg/kg p.o. In contrast to β_2-adrenergic agonists, no tachyphylaxis occurred after repeated treatment with SSR126768A, showing marked superiority over the commonly used tocolytics. Together, these data argue for a crucial role for the OTR in parturition, at least in rodents, and support findings obtained in knockout OT and prostaglandin $F_{2\alpha}$ receptor mice. Knockout OT mice showed defects in milk ejection, and, surprisingly, normal parturition (Nishimori et al., 1996). Female mice lacking the receptor for prostaglandin $F_{2\alpha}$ do not deliver fetuses at term, and no induction of the OTR mRNA was found in the uterus of these mice; in addition, they showed no uterine contraction after OT injection. Ovariectomy, at 19 day of pregnancy, restored induction of the OTR and caused successful delivery in these animals, suggesting an important role for the OTR in the parturition process (Sugimoto et al., 1997). Thus, these data suggest that the presence of OTR is a requisite for parturition, whereas the natural hormone OT is required for nursing and not for parturition.

Additionally, it is worth noting that SSR126768A displayed competitive antagonism for the natural hormone in all the different binding and functional models studied, and this effect is reversible by OT (data not shown); these properties are clinically relevant for the use of SSR126768A in controlling labor and parturition, because after birth, OT blockade is undesirable and OTR stimulation is required to counteract postpartum bleeding and to trigger milk ejection.

A further therapeutic indication for OTR antagonists could be dysmenorrhea because both OT and AVP have been involved in painful on menstruation. This pain is believed to result from uterine contractions, vasospasm, and ischemia, mainly mediated by OT/AVP and also prostaglandin secreted in the endometrium (Akerlund, 2002). Thus, SSR126768A, as an OTR antagonist, would be a likely candidate for the treatment and prevention of dysmenorrhea.

In conclusion, SSR126768A is a potent, highly selective OTR antagonist with good oral bioavailability and duration of action. It displays powerful uterine-relaxing properties in vitro and in vivo in various rat and human models. SSR126768A represents a promising tocolytic molecule for the acute and chronic management of preterm labor.

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References

Akerlund M (2002) Involvement of oxytocin and vasopressin in the pathophysiology of preterm labor and primary dysmenorrhea, in *Progress in Brain Research* (Poulain D, Oliet S, and Theodosis D eds), vol 139, pp 359–365, Elsevier, Paris, France.

Akerlund M, Bossmar T, Brouard R, Kostrzewska A, Laudanski T, Lemancewicz A, Serradeil-Le Gal C, and Steinwall M (1999) Receptor binding of oxytocin and vasopressin antagonists and inhibitory effects on isolated myometrium from preterm and term pregnant women. *Br J Obstet Gynaecol* **106:**1047–1053.

Akerlund M, Stromberg P, Hauksson A, Andersen LF, Lyndrup J, Trojnar J, and Melin P (1987) Inhibition of uterine contractions of premature labour with an oxytocin analogue. Results from a pilot study. *Br J Obstet Gynaecol* **94:**1040–1044.

Allen MJ, Smith J, Hampton S, and Giles H (1997) Affinity of antagonists and endogenous peptides at human recombinant oxytocin and vasopressin receptors, in *Word Congress of Neurohypophysial Hormones*, Montreal, Canada, August 8–12, A176 (Abstr.)

Arunlakshana O and Schild H (1959) Some quantitative uses of drug antagonists. *Br J Pharmacol* **14:**48–58.

Bardou M, Loustalot C, Cortijo J, Simon B, Naline E, Dumas M, Esteve S, Croci T, Chalon P, Frydman R, et al. (2000) Functional, biochemical and molecular biological evidence for a possible beta(3)-adrenoceptor in human near-term myometrium. *Br J Pharmacol* **130:**1960–1966.

Bell IM, Erb JM, Friedinger RM, Gallicchio SN, Guare JP, Guidotti MT, Halpin RA, Hobbs DW, Homnick CF, Kuo MS, et al. (1998) Development of orally active oxytocin antagonists: studies on 1-(1-{4-[1-(2-methyl-1-oxidopyridin-3-ylmethyl)piperidin-4-yloxyl-2-methoxybenzoyl}piperidin-4-yl)-1,4-dihydrobenz[*d*][1,3]oxazin-2-one (L-372,662) and related pyridines. *J Med Chem* **41:**2146–2163.

Carbillon L, Seince N, and Uzan M (2001) Myometrial maturation and labour. *Ann Med* **33:**571–578.

Carbonne B, Cabrol D, Clerget MS, and Germain G (1998) Effects of nomegestrol acetate on spontaneous and sulprostone-induced uterine contractions in pregnant cynomolgus monkeys monitored by telemetry. *Am J Obstet Gynecol* **178:**150–155.

Chen DL, Chan WY, and Manning M (1994) Agonist and antagonist specificities of decidual prostaglandin-releasing oxytocin receptors and myometrial uterotonic oxytocin receptors in pregnant rats. *J Reprod Fertil* **102:**337–343.

Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* **22:**3099–3108.

Cirillo R, Gillio Tos E, Schwarz MK, Quattropani A, Scheer A, Missotten M, Dorbais J, Nichols A, Borrelli F, Giachetti C, et al. (2003) Pharmacology of (2S,4Z)-N-[(2S)-2-hydroxy-2-phenylethyl]-4-(methoxyimino) -1-[(2′-methyl[1,1′-biphenyl]-4-yl)carbonyl]-2-pyrrolidinecarboxamide, a new potent and selective nonpeptide antagonist of the oxytocin receptor. *J Pharmacol Exp Ther* **306:**253–261.

Demarest KT, Hahn DW, Ericson E, Capetola RJ, Fuchs AR, and McGuire JL (1989) Profile of an oxytocin antagonist, RWJ 22164 for treatment of preterm labor in laboratory models of uterine contractility. *Am J Perinatol* **6:**200–204.

El Alj A, Winer N, Lallaoui H, Delansorne R, Ferre F, and Germain G (1993) Progesterone and mifepristone modify principally the responses of circular myo-

metrium to oxytocin in preparturient rats: comparison with responses to acetylcholine and to calcium. *J Pharmacol Exp Ther* **265:**1205–1212.
Elands J, Barberis C, Jard S, Tribollet E, Dreifuss J-J, Bankowski K, Manning M, and Sawyer H (1987) ^{125}I-Labelled d(CH2)5[Tyr(Me)2, Thr4,Tyr-NH29]OVT: a selective oxytocin receptor ligand. *Eur J Pharmacol* **147:**197–207.
Fuchs AR, Fuchs F, Husslein P, and Soloff MS (1984) Oxytocin receptor in the human uterus during pregnancy and parturitior. *Am J Obstet Gynecol* **150:**734–741.
Gimpl G and Fahrenholz F (2001) The oxytocin receptor system: structure, function and regulation. *Physiol Rev* **81:**629–683.
Goodwin TM and Zograbyan A (1998) Oxytocin receptor antagonists. Update. *Clin Perinatol* **25:**859–871.
Grynkiewicz G, Poenie M, and Tsien RY (1985) A new generation of Ca2+ indicators with greatly improved fluorescence properties. *J Biol Chem* **260:**3440–3450.
Hall M., Danielian P., and Lamont R. 1997. *The Importance of Preterm Birth* (Elder M, Lamont R, and Romero R eds) pp 1–28, Churchill Livingstone, New York.
Kawamata M, Mitsui-Saito M, Kimura T, Takayanagi Y, Yanagisawa T, and Nishimori K (2003) Vasopressin-induced contraction of uterus is mediated solely by the oxytocin receptor in mice, but not in humans. *Eur J Pharmacol* **472:**229–234.
Kobayashi M, Akahane M, Minami K, Moro M, Ajisawa Y, Inoue Y, and Kawarabayashi T (1999) Role of oxytocin in the initiation of term and preterm labor in rats: changes in oxytocin receptor density and plasma oxytocin concentration and the effect of an oxytocin antagonist, L-366,509. *Am J Obstet Gynecol* **180:**621–627.
Manning M, Stoev S, Cheng LL, Wo NC, and Chan WY (2001) Design of oxytocin antagonists, which are more selective than atosiban. *J Pept Sci* **7:**449–465.
McLean M, Bisits A, Davies J, Woods R, Lowry P, and Smith R (1995) A placental clock controlling the length of human pregnancy. *Nat Med* **1:**460–463.
McLean M, Walters WA, and Smith R (1993) Prediction and early diagnosis of preterm labor: a critical review. *Obstet Gynecol Surv* **48:**209–225.
Mitchell BF and Schmid B (2001) Oxytocin and its receptor in the process of parturition. *J Soc Gynecol Investig* **8:**122–133.
Nishimori K, Young LJ, Guo Q, Wang Z, Insel TR, and Matzuk MM (1996) Oxytocin is required for nursing but is not essential for parturition or reproductive behavior. *Proc Natl Acad Sci USA* **93:**11699–11704.
Pettibone DJ, Kishel MT, Woyden CJ, Clineschmidt BV, Bock MG, Freidinger RM, Veber DF, and Williams PD (1992) Radioligand binding studies reveal marked species differences in the vasopressin V1 receptor of rat, rhesus and human tissues. *Life Sci* **50:**1953–1958.
Romero R, Sibai BM, Sanchez-Ramos L, Valenzuela GJ, Veille JC, Tabor B, Perry KG, Varner M, Goodwin TM, Lane R, et al. (2002) An oxytocin receptor antagonist (atosiban) in the treatment of preterm labor: a randomized, double-blind, placebo-controlled trial with tocolytic rescue. *Am J Obstet Gynecol* **182:**1173–1183.
Schwarz MK and Page P (2003) Preterm labour: an overview of current and emerging therapeutics. *Curr Med Chem* **10:**1441–1468.
Serradeil-Le Gal C, Lacour C, Valette G, Garcia G, Foulon L, Galindo G, Bankir L, Pouzet B, Guillon G, Barberis C, et al. (1996) Characterization of SR 121463A, a highly potent and selective, orally active vasopressin V2 receptor antagonist. *J Clin Investig* **98:**2729–2738.
Serradeil-Le Gal C, Raufaste D, Double-Cazanave E, Guillon G, Garcia C, Pascal M, and Maffrand JP (2000) Binding properties of a selective tritiated vasopressin V2 receptor antagonist, [3H]-SR121463. *Kidney Int* **58:**1613–1622.
Serradeil-Le Gal C, Wagnon J, Garcia C, Lacour C, Guiraudou P, Christophe B, Villanova G, Nisato D, Maffrand JP, and Le Fur G (1993) Biochemical and pharmacological properties of SR 49059, a new, potent, nonpeptide antagonist of rat and human vasopressin V1a receptors. *J Clin Investig* **92:**224–231.
Serradeil-Le Gal C, Wagnon J, Simiand J, Griebel G, Lacour C, Guillon G *et al* (2002) Characterization of (2S,4R)-1-[5-chloro-1-[(2,4-dimethoxyphenyl)sulfonyl]-3-(2-methoxy-phenyl) -2-oxo-2,3-dihydro-1H-indol-3-yl]-4-hydroxy-N,N-dimethyl-2-pyrrolidine carboxamide (SSR149415), a selective and orally active vasopressin V1b receptor antagonist. *J Pharmacol Exp Ther* **300:**1122–1130.
Smith R, Mesiano S, and McGrath S (2002) Hormone trajectories leading to human birth. *Regul Pept* **108:**159–164.
Sugimoto Y, Yamasaki A, Segi E, Tsuboi K, Aze Y, Nishimura T, Oida H, Yoshida N, Tanaka T, Katsuyama M, et al. (1997) Failure of parturition in mice lacking the prostaglandin F receptor. *Science (Wash DC)* **277:**681–683.
The Worldwide Atosiban versus Beta-Agonists Study Group (2003) Effectiveness and safety of the oxytcin antagonist atosiban versus beta-adrenergic agonists in the treatment of preterm labour. *Br J Obstet Gynaecol* **108:**133–142.
Vatish M and Thornton S (2002) Oxytocin antagonists. *Exp Opin Ther Patents* **12:**1403–1406.
Williams PD, Clineschmidt BV, Erb JM, Freidinger RM, Guidotti MT, Lis EV, Pawluczyk JM, Pettibone DJ, Reiss DR, Veber DF, et al. (1995) 1-(1-[4-[(N-Acetyl-4-piperidinyl)oxy]-2-methoxybenzoyl]piperidin-4-yl)-4H-3,1-benzoxazin-2(1H)-one (L-371,257): a new, orally bioavailable, non-peptide oxytocin antagonist. *J Med Chem* **38:**4634–4636.
Wilson RJ, Allen MJ, Nandi M, Giles H, and Thornton S (2001) Spontaneous contractions of myometrium from humans, non-human primate and rodents are sensitive to selective oxytocin receptor antagonism in vitro. *Br J Obstet Gynaecol* **108:**960–966.

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