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Title Page

TAS-303, a novel selective norepinephrine reuptake inhibitor that increases urethral pressure in rats, indicating its potential as a therapeutic agent for stress urinary incontinence

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Running Title Page

Running Title: TAS-303, a novel NRI, increases urethral pressure in rats

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Abbreviations: DAT, dopamine transporter; DBP, diastolic blood pressure; EUS, external urethral sphincter; HR, heart rate; LPP, leak point pressure; MBP, mean blood pressure; MUI, mixed urinary incontinence; NE, norepinephrine; NET, norepinephrine transporter; NRI, norepinephrine reuptake inhibitor; OAB, overactive bladder; P-gp, P-glycoprotein; QOL, quality of life; SBP, systolic blood pressure; S.E.M, standard error of the mean; SERT, serotonin transporter; SNRI, serotonin norepinephrine reuptake inhibitor; SUI, stress urinary incontinence; TCA, tricyclic antidepressant; UI, urinary incontinence; UUI, urge urinary incontinence; VD, vaginal distension

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Abstract

Stress urinary incontinence (SUI) is characterized by involuntary leakage associated with exertion, effort, sneezing, coughing, or lifting. Duloxetine, a serotonin norepinephrine reuptake inhibitor, is approved for the treatment of patients with SUI in some European countries, but not in the USA. There is currently no globally approved pharmacological drug for the treatment of SUI patients. Therefore, a new pharmacological treatment option is required. TAS-303 is a novel small-molecule selective norepinephrine reuptake inhibitor that displays significant norepinephrine transporter (NET) inhibitory activity toward the serotonin or dopamine transporters. In this report, we describe the pharmacological properties of TAS-303 and its effects on urethral function, using preclinical *in vitro* and *in vivo* studies. Radioligand-binding studies showed that TAS-303 selectively and potently inhibited [^{3}H]norepinephrine binding to the human NET. Oral administration of TAS-303 (3 mg/kg) significantly increased norepinephrine levels in the plasma, whereas it did not significantly affect epinephrine, dopamine, and serotonin levels. TAS-303 (0.3, 1, and 3 mg/kg) dose-dependently increased basal urethral pressure in normal rats and leak point pressure in vaginal distention rats, exhibiting a maximal effect comparable to duloxetine. In the forced swimming test, TAS-303 (100 mg/kg) showed no significant effects on immobility time in rats, raising the possibility that this agent would have minimal central nervous system side effects at an effective dose for urethral function. These results demonstrate that TAS-303 has therapeutic potential for the treatment of patients with SUI.

Introduction

Urinary incontinence (UI) is defined as an involuntary leakage of urine and may cause adverse effects on social interactions and psychological health (Sarkar and Ritch, 2000). UI can be classified into three groups: urge urinary incontinence (UUI), stress urinary incontinence (SUI), and mixed urinary incontinence (MUI). UUI is characterized by a sudden desire to void with involuntary voiding and leakage of significant amounts of urine, and is frequently caused by an overactive bladder (OAB). Several pharmacologic treatment options, such as muscarinic M_3 receptor antagonists and β_3-adrenoreceptor agonists, are available for OAB. These are recognized to be effective in the improvement of OAB symptoms and have a good safety profile (Maman et al., 2014). SUI is characterized by involuntary leakage on effort or exertion, or on sneezing or coughing (Abrams et al., 2002). SUI commonly occurs in adults, particularly woman. It has been reported that the prevalence of SUI is 21.5 - 38.2% in woman older than 20 years and 52.5% for those older than 50 years (Hannestad et al., 2000; Minassian et al., 2008; Coyne et al., 2012). Previously, some SUI studies have attempted to assess the impact of incontinence on quality of life (QOL). In these studies, it was demonstrated that woman suffering from severe SUI hesitated to go outdoors (Grimby et al., 1993; Swithinbank and Abrams, 1999). While SUI is not considered a life-threatening disease, its impact on patients' QOL is often devastating and results in social isolation for many. The main cause of SUI is thought to be the damage of muscles and nerves near the urethral sphincter during pregnancy and delivery, leading to the impairment of urethral resistance. In this context, the basic concept of SUI treatment is to increase urethral resistance. Current treatment options for SUI are as follows: surgical therapy, physical therapy, and pharmacological therapy. Surgical therapy is usually conducted for patients with moderate to severe SUI. A review of the literature demonstrated that in women 65 years or younger who underwent mid urethral sling procedures, SUI cure rates ranged from 73% to 95% (Gerten et

al., 2008). However, surgical intervention is invasive and has been associated with vaginal erosion, infection, and *de novo* urge symptoms (Gerten et al., 2008). In physical therapy, postpartum pelvic floor muscle training can be effective for patients with mild to moderate SUI (Mørkved and Bø, 2000). However, in one study, the majority of women (61%) prescribed pelvic floor muscle training for SUI were non-compliant, possibly discouraged by the slow response with the training, and thus failed to dedicate the 15–20 weeks recommended to assess their response (Lagro-Janssen and Weel, 1998).

In pharmacological therapy, duloxetine, a serotonin and norepinephrine reuptake inhibitor (SNRI) has demonstrated clinical efficacy for SUI patients (Norton et al., 2002; Dmochowski et al., 2003). Duloxetine is reported to act in both the central nervous system (CNS) and the peripheral nervous system (PNS) (Thor, 2003; Miyazato et al., 2008). In the CNS, it was shown that duloxetine enhanced noradrenergic and serotonergic excitatory control of external urethral sphincter (EUS) spinal motoneurons and evoked contractions of the EUS. In the PNS, duloxetine increased norepinephrine (NE) levels at sympathetic postganglionic nerve terminals, leading to activation of peripheral adrenergic α_1 receptors in urethral smooth muscles. These actions are therefore considered to be the underlying mechanism responsible for its efficacy in women with SUI. However, duloxetine is labeled with a ‘black box warning’ about the increased risk of suicide (Friedman and Leon, 2007). It has been reported that the serotonin system is associated with the onset of the above-mentioned side effect (Oelke et al., 2006; Mann, 2013). In other words, the inhibition of serotonin transporter (SERT) by duloxetine may be involved in the increased risk of suicide. Consequently, duloxetine is not approved for the treatment of SUI in the USA. As there is no globally approved drug for the treatment of patients with SUI, a new pharmacological treatment option is required. For these reasons, a selective norepinephrine reuptake inhibitor (NRI) is a potential therapeutic drug for SUI patients that lack the side effects associated with SERT inhibition. We created the novel

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selective NRI TAS-303 hydrochloride [4-piperidinyl 2,2-diphenyl-2-(propoxy-*1,1,2,2,3,3,3-d*$_7$)acetate hydrochloride]. In this study, we report that TAS-303 selectively inhibited norepinephrine transporter (NET) over SERT or dopamine transporter (DAT) and also inhibited the uptake of [^{3}H]norepinephrine *in vitro*. We also demonstrate that TAS-303 exhibited a dose-dependent increase in basal urethral pressure in normal rats and leak point pressure (LPP) in vaginal distention (VD) rats *in vivo*.

Materials and Methods

Ethics Statement

All animal experiments were executed according to the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health and approved by the local Committee of Animal Use and Care of Taiho Pharmaceutical Co. Ltd (Tsukuba, Japan). Female Sprague-Dawley rats at 10 – 13 weeks of age (Charles River Japan, Kanagawa, Japan) were used for this study and housed in a controlled environment (24±1°C, 12 h light/dark cycle) with free access to food and water.

Chemicals

TAS-303 hydrochloride was synthesized by Taiho Pharmaceutical Co. Ltd. The TAS-303 synthesis method was previously described (Publication No. WO 2013/115077, published in 2013). The chemical structure of TAS-303 is shown in Fig. 1. Duloxetine hydrochloride was obtained from Bepharm Ltd. (Shanghai, China). L-(−)-Norepinephrine (+)-bitartrate salt monohydrate was purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan). Imipramine hydrochloride was obtained from Sigma-Aldrich.

Transporter Binding

Dilutions of each test compound were made for concentrations ranging from 3 – 3000 nM for TAS-303, and 0.3 – 300 nM for duloxetine. Each assay was performed according to the protocols of Eurofins Pharma Discovery Services Taiwan Ltd., Taipei, Taiwan. Briefly, dog kidney MDCK cells expressing human NET were used for binding assays in assay buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 µM leupeptin, 10 µM PMSF). A 40-mg aliquot of membranes was incubated with 0.2 nM [^{125}I]RTI-55 for 3 hr at 4°C. Non-specific binding was estimated in the presence of the tricyclic antidepressant (TCA) desipramine at 10 µM. Membranes were filtered and washed 3 times, and the filters were then counted to determine the specific binding of [^{125}I]RTI-55. For binding to SERT, HEK-293 cell membranes stably

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transfected with a plasmid encoding the human serotonin transporter were prepared in assay buffer using standard techniques. A 9-mg aliquot of membranes was incubated with 0.4 nM [^{3}H]paroxetine for 60 min at 25°C. Non-specific binding was estimated in the presence of the TCA imipramine at 10 µM. Membranes were filtered and washed 3 times, and the filters were counted to determine [^{3}H]paroxetine specific binding. DAT binding assays were conducted using CHO-S cells expressing human DAT in assay buffer. A 40-mg aliquot of membranes was incubated with 0.15 nM [^{125}I]RTI-55 for 3 hr at 4°C. Non-specific binding was estimated in the presence of the dopamine reuptake inhibitor nomifensine at 10 µM. Membranes were filtered and washed 3 times, and the filters were then counted to determine [^{125}I]RTI-55 specific binding. Competition studies were carried out to determine the potencies of unlabeled compounds to displace [^{125}I]RTI-55, [^{3}H]paroxetine, or [^{125}I]RTI-55 for NET, SERT, or DAT binding to membranes, respectively. All assays were performed in triplicate.

Monoamine Uptake

Each assay was performed according to the protocols of Eurofins Pharma Discovery Services Taiwan Ltd. Briefly, NE uptake was assessed in MDCK cells expressing human recombinant NET that were plated overnight. Test compounds (TAS-303, 3 - 3000 nM; Duloxetine, 0.3 - 300 nM) were pre-incubated with cells (2×10^{5} cells/mL) in assay buffer (5 mM Tris-HCl, 7.5 mM HEPES, 120 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5 mM D-glucose, 1 mM ascorbic acid, pH 7.1) for 20 min at 25°C; 25 nM [^{3}H]norepinephrine was then added and incubated for an additional 15 min. After the assay buffer was removed, the cells were washed in assay buffer to remove free [^{3}H]norepinephrine. A lysate was obtained from the solubilized cells and counted to determine [^{3}H]norepinephrine uptake. Reduction of [^{3}H]norepinephrine uptake by 50 percent or more (≥50%), relative to 10 µM desipramine, indicated significant inhibitory activity. Serotonin uptake was assessed in HEK-293 cells expressing human recombinant SET that were plated overnight. Test compounds were

pre-incubated with cells (1×10^5 cells/mL) in assay buffer for 20 min at 25°C; 65 nM [^{3}H]serotonin was then added and incubated for an additional 15 min. Intracellular [^{3}H]serotonin is trapped on the filters. The filters were then rinsed with assay buffer and counted to determine [^{3}H]serotonin uptake. Reduction of [^{3}H]serotonin uptake by 50 percent or more (≥50%), relative to 1 μM fluoxetine, indicated significant inhibitory activity. Dopamine uptake was assessed in CHO-S/hDAT clone 28 cells expressing human recombinant DAT that were plated overnight. Test compounds were pre-incubated with cells (4×10^4 cells/mL) in assay buffer for 20 min at 25°C; 50 nM [^{3}H]dopamine was then added and incubated for an additional 10 min. After the assay buffer was removed, the cells were washed in assay buffer to remove free [^{3}H]dopamine. A lysate was obtained from the solubilized cells and counted to determine [^{3}H]dopamine uptake. Reduction of [^{3}H]dopamine uptake by 50 percent or more (≥50%), relative to 10 μM nomifensine, indicated significant inhibitory activity. If significant inhibition of uptake was observed, the possibility of compound-induced cytotoxicity was evaluated by applying the same concentrations of compounds described above to a separate group of untreated cells.

Measurement of Isolated Urethral Contractions

Female Sprague-Dawley rats 11 – 13 weeks of age were euthanized by decapitation and the urethra was isolated. The proximal urethra was cut open and then cut in the traverse direction. Specimens 1.5 mm in width and 2 mm in length were prepared. The prepared specimens were transferred to a 37°C organ bath (10 mL) filled with Krebs-Henseleit solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 10 mM glucose) saturated with 95% O_2 - 5% CO_2 gas. One end of the specimen was connected to an FD pick-up transducer (TB-612T; Nihon Kohden Co., Ltd., Tokyo, Japan) and the other end was fixed to the organ bath. Urethral tension was recorded and analyzed using a PowerLab 16/30 (AD Instrument Pty Ltd., Sydney, Australia) via an FD pick-up transducer and strain

pressure amplifier (AP-601G; Nihon Kohden). NE-induced urethral contractions were assessed in reference to the previously described method (Pérez-Martínez et al., 2011). A resting tension of 1.0 g was applied to the isolated urethras and the specimens were equilibrated for approximately 40 min. Ultra-pure water (100 μL) was added to the organ bath. Ten min later, 100 μL of a 100 mM NE solution was added to the vessel and NE-induced urethral contractions were measured. Urethral specimens were washed with Krebs-Henseleit solution, and recovery of tension to the original equilibration level was confirmed 40 min after the previous measurement of NE-induced contractions. Then, 100 μL of ultra-pure water was added to the organ bath. The final concentrations of NE in the organ bath were 1 nM to 1 mM. This procedure was repeated until the urethral specimen stabilized, and the data obtained after stabilization were used as the control data. After measurement of the control data, the urethral specimen was washed with Krebs-Henseleit solution. Recovery of resting tension to the original equilibration level was confirmed 50 min after previous measurement of NE-induced contractions. A series of solutions (5 concentrations) of each test substance was assessed from low concentration to high concentration using the same specimen. The NE (1 mM)-induced maximum contraction when examined with vehicle alone (ultra-pure water) prior to evaluation of test substances as control data was designated as 100% contraction, and the urethral contractions for each test solution were expressed as relative values (%). The EC_{50} of NE was obtained from a sigmoidal curve under variable E_{max} conditions.

Determination of TAS-303 Levels in Rat Plasma

A single dose of TAS-303 (1, 3, or 10 mg/kg) was administrated by gavage. Blood samples were collected from the jugular vein into heparinized tubes at 0, 0.5, 1, 2, 4, 8, 12, and 24 hr after administration. These samples were centrifuged at 4°C and the supernatants were collected as plasma. Plasma concentrations of TAS-303 were determined using a

high-performance liquid chromatographic method combined with tandem mass spectrometry.

Measurement of Monoamine Levels in Plasma

Female Sprague-Dawley rats (10 weeks of age) were divided into a control group (N=10) and a TAS-303 group (N=9). Rats were treated once daily with distilled water or TAS-303 (3 mg/kg) orally. Rats were anesthetized by intraperitoneal injection of 1.2 g/kg urethane 30 min after the final administration. Blood samples were collected from the inferior vena cava into tubes containing EDTA as the anti-coagulant. A portion of the blood was kept at -70°C for serotonin analysis. The remaining blood was centrifuged at 4°C and the supernatant was collected as plasma. The plasma samples were stored in a freezer at -70°C for the analysis of epinephrine, norepinephrine, and dopamine. Monoamine levels were measured by BML Inc. (Tokyo, Japan).

Measurement of Urethral Pressure

Female Sprague-Dawley rats at 11 weeks of age were anesthetized by intraperitoneal injection of 1.2 g/kg urethane. After confirming the loss of extremity reflex, an abdominal median incision was performed. Both ureters were ligated with a 4-0 silk ligature, and the ureter was cut at the ligated position near to the kidney. Then, the urethra was ligated tightly with a 4-0 silk ligature. The dome of the bladder was cut open and a catheter (PE-50; Nippon Becton, Dickinson and Company, Tokyo, Japan) was placed within, and the opening of the bladder was then sutured with a purse string suture. A three-way stopcock was attached to the other end of the catheter and the stream was divided into two paths. One was connected to a pressure transducer (DX-360; Nihon Kohden) and the other was connected to a syringe filed with physiological saline and connected to a continuous infusion device (KDS200; KD Scientific Inc., Holliston, MA, USA). An additional catheter was inserted into the urethra from the external urethral orifice. A three-way stopcock was attached to the outer end of this catheter and the stream was divided into two paths. One was connected to a pressure

transducer and the other was connected to a syringe as described above. After catheterization, physiological saline was infused into the bladder at a rate of 10 mL/hr via a micro-syringe pump connected to the bladder on a heating board (PS-53; Sakura Finetek Japan Co., Tokyo, Japan). The infusion was stopped when rhythmic contractions of the bladder were confirmed. Physiological saline was then infused into the urethra at a rate of 3 mL/h and the inner pressure of the bladder and urethral pressure were measured with a pressure amplifier (AP-641G; Nihon Kohden) connected to a pressure transducer, and recorded using PowerLab 16/30. TAS-303 at 0.3, 1, or 3 mg/kg or vehicle (ultra-pure water) was administered by gavage to rats 30 min prior to urethane anesthesia. For evaluation of duloxetine (1 mg/kg), rats were subjected to the operation described above and treated intravenously after equilibration of intravesical pressure and intraurethral pressure. Urethral pressure was analyzed by its waveform using Chart v5.2.2 (AD Instrument Pty Ltd.). The primary evaluation point was set at the urethral baseline pressure, and the effects of duloxetine were assessed relative to the baseline values. Following oral treatment, individual urethral baseline pressure was calculated as an average pressure during a period of 30 min from 1 hr after administration. For intravenous administration, individual urethral baseline pressure was calculated as an average pressure during the 30-min period following administration.

Measurement of LPP

Female Sprague-Dawley rats 11 weeks of age were anesthetized by intraperitoneal injection of 50 mg/kg pentobarbital. After confirming the loss of body reflex, a balloon catheter (JU-LB1005; Terumo Corporation, Tokyo, Japan) was inserted into the vagina. A 4-mL aliquot of physiological saline was injected into the balloon to expand the catheter. The rats were then placed on a heating board warmed to 37ºC for 3 hr to maintain vaginal distention. Next, the distention was stopped and the rats were returned to normal housing conditions with free access to food and water. LPP was assessed 4 days after VD model preparation.

Rats were anesthetized by intraperitoneal injection of 1.0 - 1.2 g/kg urethane. Rats were placed in a prone position and the spinal cord was completely severed between Th9 and Th10. The rats were then inverted to a supine position and an abdominal median incision was performed. A vinyl-tube (SV45; Natsume Seisakusho Co., Ltd., Tokyo, Japan) was then inserted and indwelled in the bladder from the dome of bladder. A three-way stopcock was attached to the other end of the catheter; one path was connected to a syringe filed with physiological saline, and the other was connected to a pressure transducer. Next, the bladder was emptied and 200 – 350 μL of physiological saline was injected into the bladder. After instillation of saline, a gentle slow manual pressure increase was applied to the rat's abdomen by a trained investigator until urethral leakage occurred, stimulating a mild Credé maneuver as previously described (Woo et al., 2009). Intravesical pressure at the occurrence time of urine leakage was designated as LPP. Intravesical pressure was measured by a pressure amplifier connected to a pressure transducer, and recorded and analyzed using PowerLab 16/30. TAS-303 at 0.3, 1, or 3 mg/kg or vehicle (ultra-pure water) was orally administered to the rats 30 min prior to urethane anesthesia, and the animals were subjected to surgery for LPP measurement as described above. LPP was measured 1 hr after dosing. For intravenous administration, pre-LPP values were determined prior to administration of vehicle (physiological saline) or 1 mg/kg duloxetine, and LPP was measured again 5 min after intravenous administration. The measurement of LPP was conducted in a blinded manner.

Forced Swimming Test (FST)

The FST was conducted according to the previously described method (Porsolt et al., 1978). A cylindrical acrylic tank (20 cm in diameter and 50 cm in height) was filled with water (at room temperature) to a depth of 25 cm. For acclimatization to the test system, 11-week-old female Sprague-Dawley rats were subjected to a forced swimming trial for 15 min one day prior to administration of test substances. The immobility time of rats in the water during the

15-min acclimatization trial was measured. Any rat showing an immobility time of less than 180 sec during the 15 min-trial, indicating a lack of apparent depressive condition, was excluded from the study. Based on the immobility time in the trial, rats were categorized as follows. “Swimming” was designated as “the state when rats were moving both fore- and rear-extremities”, and “the time of not swimming” was designated as “immobility time”. The day following the swimming acclimatization trial, vehicle, TAS-303 (10, 30, 100 mg/kg), or imipramine (60 mg/kg) was orally administered to rats. One hr after dosing, individual rats were placed in the tank and the FST was conducted for 5 min. Immobility time was measured as described above. Evaluation of the results was conducted in a blinded manner.

Statistical Analysis

Results are reported as the mean ± S.E.M. Statistical analysis was carried out using a student’s *t*-test or Williams’ test. A p-value < 0.05 was considered to show statistical significance. All data analyses were performed using SAS version 9.2 and EXSUS version 8.0.0 (CAC Exicare Corporation, Tokyo, Japan).

Results

Affinity to Monoamine Transporters and Inhibition of Monoamine Uptake by TAS-303

The inhibitory potential of TAS-303 and duloxetine on ligand binding to monoamine transporters was assessed using human NET, human SERT and human DAT expressing cells (Table 1). TAS-303 selectively inhibited binding of the NE transporter ligand to membranes from cells transfected with human NET relative to the serotonin and dopamine transporters. Duloxetine selectively inhibited binding of NE and serotonin transporters relative to the dopamine transporter. In functional cell-based assays, the inhibitory effects of TAS-303 and duloxetine on the uptake of NE, serotonin and dopamine were also evaluated (Figure 2, Table 2). TAS-303 selectively inhibited the cellular uptake of [^{3}H]norepinephrine relative to [^{3}H]serotonin and [^{3}H]dopamine. Duloxetine selectively inhibited the uptake of [^{3}H]norepinephrine and [^{3}H]serotonin relative to [^{3}H]dopamine.

Effect of TAS-303 on Norepinephrine-induced Contractions of Rat Isolated Urethra

The effect of TAS-303 on NE-induced contractions of isolated urethral strips was assessed to confirm the efficacy of TAS-303. The reproducibility of consecutive NE-induced contraction-response curves on the same tissue was evaluated in the presence of the vehicle for TAS-303 and duloxetine (ultrapure water). The EC_{50} values for the concentration-response curves for vehicle are shown in Table 3. Five consecutive vehicle treatments produced no significant change in EC_{50} values through 6 trials (Figure 3, Table 3). Duloxetine shifted the concentration-response curve to the left. Significant differences were observed at concentrations of 10 nM or greater (P<0.05 for 10 nM, P<0.01 for more than 30 nM). TAS-303 also shifted the concentration-response curve to the left. A significant difference was observed at a concentration of 100 nM or greater (P<0 .01).

Pharmacokinetic Profile of TAS-303 in Rats

The PK of TAS-303 was assessed in female Sprague-Dawley rats after oral administration

(Table 4). TAS-303 (1, 3, or 10 mg/kg) was absorbed with a time to peak concentration (T_{max}) of 0.75 ± 0.29, 0.75 ± 0.29 or 1.25 ± 0.59 hr, respectively, in rats. The mean maximum plasma concentration (C_{max}) of TAS-303 increased in a dose-dependent manner in rats from 11.7 ± 5.0, 49.1 ± 13.9, or 453.0 ± 28.8 nM for doses of 1, 3, or 10 mg/kg, respectively.

Plasma Monoamine Concentration

To determine the functional effects of TAS-303, rats were administered TAS-303 and its ability to increase plasma monoamines was evaluated (Figure 4). Plasma norepinephrine levels were significantly higher in the TAS-303 group than in the vehicle group. In contrast, there were no differences in plasma epinephrine, dopamine, or serotonin levels between the two groups.

Effect of TAS-303 on Urethral Pressure in Rats

A previous study showed that intravenous injection of duloxetine (1 mg/kg) significantly increased the basal urethral pressure in rats (Kamo and Hashimoto, 2007). To assess the pharmacological potential of TAS-303 for regulating urethral resistance *in vivo*, the basal urethral pressure of rats was measured after oral administration (Figure 5). Intravenous administration of physiological saline induced no apparent changes in urethral baseline pressure (data not shown). Intravenous administration of duloxetine (1 mg/kg) significantly increased urethral baseline pressure by 15% compared to the vehicle group ($P<0.05$). Oral administration of TAS-303 (0.3, 1, and 3 mg/kg) resulted in a dose-dependent increase in urethral pressure. At greater than 1 mg/kg of TAS-303, urethral baseline pressure significantly increased compared to the vehicle group ($P<0.05$), and increased by 38% at a dose of 3 mg/kg.

Effect of TAS-303 on LPP in VD Model Rats

SUI has been reported to mainly occur as a result of pregnancy and delivery. The VD model was selected because it is commonly used to simulate vaginal delivery, and imitates the

urethral damage occurring after delivery (Lin et al., 1998; Cannon et al., 2002). A previous report showed that intravenous injection of 1 mg/kg duloxetine significantly increased sneeze-induced LPP in VD rats (Miyazato et al., 2009). To investigate the effect of TAS-303 on urethral function in an SUI model, LPP in the rat VD model was measured after oral administration (Figure 6). LPP was defined as the intravesical pressure at the occurrence of incontinence induced by abdominal pressure loading, and the experiment was conducted in a blinded manner. LPP of VD rats was 32.5 ± 1.6 cmH_2O and was significantly lower than that of sham-operated rats (50.6 ± 2.6 cmH_2O, $P<0.01$), indicating successful establishment of the VD model. Intravenous administration of duloxetine (1 mg/kg) significantly increased LPP by 20% compared to the vehicle group ($P<0.01$). Oral administration of TAS-303 (0.3, 1, and 3 mg/kg) resulted in a dose-dependent increase in LPP. At greater than 1 mg/kg TAS-303, LPP was significantly increased compared to the vehicle group ($P<0.05$), and increased by 26% at a dose of 3 mg/kg.

Effect of TAS-303 on Blood Pressure and Heart Rate in dogs

It has been reported that antidepressants are associated with the potential cardiovascular risks (Mago et al., 2014). Thus, effects of TAS-303 on cardiovascular parameters in dogs were assessed to clarify whether TAS-303 would have the risk of cardiovascular side effects (Supplemental Figure 1). The dog is selected because this is one of the most generally used animal models for assessing the effects of drugs on blood pressure and heart rate. Time course of the effects of a single oral administration of TAS-303 (1, 3, or 10 mg/kg) on systolic blood pressure (SBP), diastolic blood pressure (DBP), mean blood pressure (MBP), and heart rate (HR) was evaluated in conscious dogs. Pre-drug values for SBP, DBP, and MBP in the vehicle-treated group were 137 ± 4, 90 ± 8, and 106 ± 6 mm Hg, respectively, these values being similar to those in the each TAS-303-treated group. Pre-drug value for HR in the vehicle-treated group was 79 ± 6 beats/min, which was similar to that in the each

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TAS-303-treated group. No significant difference between the TAS-303 group (at each dose) and the vehicle group was detected in the evaluation of SBP, DBP, MBP, and HR.

Effect of TAS-303 on Immobility Time of Rats in the FST

The FST is one of the most frequently used animal models for assessing antidepressant-like effects of centrally acting antidepressants, such as selective serotonin reuptake inhibitors, SNRIs, and TCAs. It is well established that treatment with such drugs reduces the duration of immobility in the FST (Rénéric and Lucki, 1998; Guan et al., 2004). To clarify whether TAS-303 affects the central nervous system, the immobility time of rats in the FST was assessed. The effects of treatment with TAS-303 and the TCA imipramine on immobility time in the FST are shown in Figure 7. Imipramine was selected as a positive control, as the effect of this agent on immobility time in the FST is well described (Guan et al., 2014). The immobility time in the imipramine group was significantly lower than that in the vehicle group ($P<0.01$). In contrast, no significant difference between the TAS-303 group (at each dose) and the vehicle group was detected.

Discussion

In this paper, we report on the pharmacological properties of the novel NRI inhibitor TAS-303. The pharmacological profile of TAS-303 (potency, selectivity, and *in vivo* efficacy) and its PK profile were evaluated. To further support the rationale for norepinephrine reuptake inhibition, we also examined the effect of TAS-303 on plasma norepinephrine levels in rats. Moreover, we estimated the antidepressant-like effect of TAS-303 using the FST. These studies characterize TAS-303 as an orally available, potent, and selective NRI with a lesser risk of central nervous system adverse events.

We investigated the direct effect of TAS-303 on monoaminergic and cholinergic receptors. The percentage agonistic activity of TAS-303 (10 μM) on the adrenergic α_{1A}, α_{1B} receptor, or 5-HT_{2c} receptor was -4%, 4%, or 0%, respectively. The ligand-binding inhibition of TAS-303 (10 μM) on the adrenergic α_{1D} receptor, nicotinic acetylcholine receptor, or 5-HT_{1A} receptor was 7%, 12%, 7%, respectively (data not shown). Therefore, TAS-303 is considered to have almost no direct effect on the receptors associated with urethral smooth muscle and EUS contraction. Among the three monoamine transporters (NET, SERT, and DAT), TAS-303 selectively inhibited NET (Ki value of 50.9 nM) and also inhibited the uptake of [^{3}H]norepinephrine (IC_{50} of 38.9 nM). Furthermore, the concentration-response curve for NE-induced urethral contractions was shifted to the left following treatment with TAS-303 in the urethral muscle strip experiment. The effect of TAS-303 on plasma monoamine levels was evaluated, and the elevation of norepinephrine level, but not the other monoamines tested, in the plasma was observed after administration of 3 mg/kg TAS-303. In the rat efficacy study, treatment with 3 mg/kg TAS-303 produced an increase in urethral pressure, where C_{max} of TAS-303 was above the IC_{50} of the *in vitro* uptake assay (38.9 nM). We have confirmed that TAS-303 is a substrate for P-gp, indicating that this agent would have a low ability to penetrate the blood-brain-barrier (data not shown). Moreover, we have confirmed that

total ^{14}C radioactivity in the plasma for orally administered ^{14}C-labelled TAS-303 (3 mg/kg) peaked at 1 hr after administration using quantitative whole-body autoradiography, and no ^{14}C radioactivity was observed in the cerebrum, cerebellum, or spinal cord (data not shown). Therefore, TAS-303 is not likely to enter the central nervous system. These findings suggest that the peripheral elevation of NE levels induced by TAS-303 administration underlies the increase in urethral pressure in rats.

Previous animal studies have suggested that NE and serotonin are involved in the neural control of lower urinary tract function (Gajewski et al., 1984; Downie and Bialik, 1988; Danuser and Thor, 1996; Espey et al., 1998; Thor et al., 1990; Conlon et al., 2009). Miyazato et al. reported that duloxetine acted in both the CNS and the PNS and stated that duloxetine seems to be superior to the NRI nisoxetine in enhancing active urethral closure, possibly because of the additional activation of the serotonergic system by duloxetine (Miyazato et al., 2008). In contrast to duloxetine, it is suggested that TAS-303 peripherally inhibited NET without affecting NET in the CNS. Concern remains about the efficacy of TAS-303 for SUI patients, because this agent has inhibitory activity against peripheral NET alone. To estimate the efficacy of TAS-303, we compared its effect to that of duloxetine on urethral resistance *in vivo*. In this study, TAS-303 significantly increased urethral pressure by 38% in normal rats. In the same assessment, the clinically approved agent duloxetine produced a 15% increase in urethral pressure. Furthermore, TAS-303 significantly increased LPP by 26% in VD rats. In the same assessment, intravenous administration of duloxetine produced a 20% increase of LPP in VD rats. Therefore, TAS-303 increased the urethral pressure of normal rats, and increased the LPP of VD rats to levels comparable to duloxetine. These results suggest that the inhibition of peripheral NET may be sufficient to increase urethral resistance. This suggestion is supported by a previous report, in which the selective NRI esreboxetine exhibited clinical efficacy in a US study of SUI patients (Klarskov et al., 2009). In addition,

Fujimori et al. evaluated the *in vivo* potency of the peripheral-selective NRI compound 12 on normal rat LPP compared to esreboxetine. Compound 12 produced an elevation in LPP and the maximum response was comparable to esreboxetine (Fujimori et al., 2015). Considering the negative CNS-penetrating property of compound 12, the increasing effect on urethral pressure in rats was suggested to be mainly mediated by the inhibition of peripheral NET. In this study, we selected 1 mg/kg as the dose of duloxetine, because this dose is often used to produce maximal effects on urethral pressure (Kamo and Hashimoto, 2007; Miyazato et al., 2015). It has been reported that the C_{max} of duloxetine after intravenous administration of radiolabelled duloxetine (5 mg/kg) to rats was 14 μM (Application Brochure, Eli Lilly Japan K.K.), and this concentration is sufficiently higher than the C_{max} (116 nM) after oral administration of the clinical dose (40 mg) in humans (Interview Form for Cymbalta, revised in 2017, Eli Lilly Japan K.K.). Thus, the plasma level of duloxetine following intravenous administration of 1 mg/kg would reach a value equivalent to or greater than the clinical dose. These findings indicate that TAS-303 has therapeutic potential as a SUI treatment. However, a limitation of this study is the difference in the administration route between TAS-303 and duloxetine, which did not allow adequate assessment of the effect of both drugs in the *in vivo* study. Accordingly, the efficacy of TAS-303 in SUI patients needs to be elucidated in clinical trials.

NE is well known to bind to adrenergic receptors in the blood vessels and myocardium, which caused increase in blood pressure and heart rate. Thus, NRIs are considered to have potential cardiovascular risks. However, previous reports have shown that in depression patients receiving a selective NRI reboxetine, there was a tendency toward orthostatic changes in blood pressure, but this was not clinically significant. (Tanum 2000; Versiani et al., 2000). Moreover, a review of the literature about cardiovascular safety profile of duloxetine have demonstrated that use of duloxetine does not appear to be associated with significant

cardiovascular risks in patients with depression, or diabetic neuropathic pain (Wernicke et al., 2007). Therefore, it is considered that NET inhibition would have little risk of clinically significant cardiovascular side effects. In this report, a single dose of TAS-303 (10 mg/kg) to dogs resulted in no significant change in blood pressure and heart rate, where C_{max} (431 nM; data not shown) of TAS-303 was above the C_{max} (49 nM) in measurement of rat urethral pressure. These findings suggested that NRIs have the selectivity for urethral efficacy over cardiovascular events. There is a need for future studies to elucidate the selectivity. That remains to be confirmed in clinical trials of TAS-303, whether increases in urethral pressure can be achieved in the absence of cardiovascular events with NET inhibition.

Antidepressants are required to be labeled with a black box warning about the increased risk of suicide (Friedman and Leon, 2007). It has been reported that the serotonin system is associated with the onset of the side effect mentioned above (Oelke et al., 2006; Mann, 2013). Consequently, duloxetine is not approved for the treatment of SUI in the USA. To assess the risk of suicide associated with TAS-303, the FST was conducted in rats. It is well established that treatment with centrally acting antidepressants, including SNRIs, and TCAs, reduce the immobility time in the FST (Guan et al., 2004). As described previously, the TCA imipramine (60 mg/kg) reduced the immobility time of rats in the FST. A previous study showed that subcutaneous administration of duloxetine at greater than 10 mg/kg significantly decreased the immobility time of rats in the FST (Rénéric and Lucki, 1998). These findings suggest that duloxetine exhibits an antidepressant-like effect in the FST. Clinically, duloxetine is often prescribed for both major depressive disorder and SUI at a dose of 40 mg/day. Therefore, it is assumed that the anti-SUI effect and antidepressant effect are produced in the same dose range. In contrast to imipramine, TAS-303 had no effect on the immobility time of rats in the FST, even at a high dose (100 mg/kg). These findings suggest that TAS-303 presents little risk of central adverse effects at an effective dose for urethral function.

TAS-303 demonstrated selective NET inhibitory activity and produced a significant increase in urethral resistance in rats, with comparable maximum effect to the clinically effective drug duloxetine, suggesting that this agent has therapeutic potential for SUI patients. Moreover, TAS-303 showed no effect on the immobility time of rats in the FST test, indicating that this agent would have a lesser risk of central adverse effects. Recently, a double-blind, single-dose, placebo-controlled crossover study of TAS-303 at a dose of 18 mg was conducted in 16 patients with SUI (Yono et al., 2017). There were no reports of serious adverse events, significant changes in blood pressure, or abnormal urinalysis results, suggesting that this agent was well tolerated. At present, a phase II randomized clinical study of TAS-303 in female patients with SUI is being conducted (NCT02906683).

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Authorship Contribution

Participated in research design: Hiroya Mizutani, Fukumitsu Sakakibara, Masahito Komuro, and Eiji Sasaki.

Conducted experiments: Hiroya Mizutani, Fukumitsu Sakakibara, and Masahito Komuro.

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Wrote or contributed to the writing of the manuscript: Hiroya Mizutani, and Fukumitsu Sakakibara.

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Figure legends

Figure 1 The chemical structure of TAS-303

Figure 2 Concentration-dependent inhibition of [^{3}H]norepinephrine, [^{3}H]serotonin, or [^{3}H]dopamine uptake by (A) duloxetine and (B) TAS-303. Inhibition of monoamine uptake in cells expressing the norepinephrine transporter, serotonin transporter, or dopamine transporter by the compounds at various concentrations was evaluated. The inhibition rate was determined from three independent experiments and data are expressed as % control specific binding. Results are shown as mean ± S.E.M. (n = 3).

Figure 3 Concentration-response curves for norepinephrine (NE) in rat isolated urethral muscle in the presence of (A) vehicle, (B) TAS-303, and (C) duloxetine. The NE (1 mM)-induced maximum contraction determined in the vehicle alone (prior to evaluation of compounds) was designated as 100% contraction, and the urethral contractions for each compound were expressed as relative values (%). Results are shown as mean + S.E.M. (n = 5 for each compound in each tissue).

Figure 4 Effect of TAS-303 (3 mg/kg) administration for 7 days on plasma concentrations of (A) epinephrine, (B) norepinephrine, (C) dopamine, and (D) serotonin in rats. Results are shown as mean + S.E.M. (n = 9-10). **; $P < 0.01$ vs. vehicle by an unpaired student's t-test.

Figure 5 Effects of duloxetine and TAS-303 on urethral baseline pressure in rats. (A) Vehicle or duloxetine (1 mg/kg) was administered intravenously, and the urethral baseline pressure was measured for 30 min after administration. (B) Vehicle or TAS-303 (0.3, 1, or 3 mg/kg) was administered orally, and the urethral baseline pressure was measured for a period of 30

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min beginning 1 hr after administration. Results are shown as mean + S.E.M. of 5 rats in each group. *$P < 0.05$ vs. vehicle (physiological saline) by a paired student's t-test. $^{\#}$ $P < 0.05$ vs. vehicle by Williams' test.

Figure 6 Effects of duloxetine and TAS-303 on leak point pressure (LPP) in vaginal distension (VD) rats. Representative traces of intravesical changes at 1 hr after administration of (A) sham, (B) vehicle or (C) TAS-303 (3 mg/kg) are shown. LPP is the intravesical pressure at the occurrence time of urine leakage (Arrow). (D) Quantification of LPP in sham or VD rats. (E) Vehicle or duloxetine (1 mg/kg) was administered intravenously, and LPP was measured at 5 min after administration. (F) Vehicle or TAS-303 (0.3, 1, or 3 mg/kg) was administered orally, and LPP was measured at 1 hr after administration. Results are shown as mean + S.E.M. of 10 rats in each group. ** $P < 0.01$ vs. vehicle (physiological saline) by a paired student's t-test. $^{\#}$ $P < 0.05$ vs. vehicle by Williams' test.

Figure 7 Effects of TAS-303 and imipramine on immobility time of rats in the forced swimming test. Measurement of immobility time was conducted at 1 hr after oral administration of vehicle, TAS-303 (10, 30, 100 mg/kg), or imipramine (60 mg/kg). Results are shown as mean + S.E.M. of 10 rats in each group. ** $P < 0.01$ vs. vehicle by an unpaired student's t-test.

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Table 1. Inhibition of monoamine transporter binding *in vitro* by TAS-303 and duloxetine

Compound	Monoamine Transporter Binding Ki ± S.E.M. (nM)			Selectivity	
	NET	SERT	DAT	SERT/NET	DAT/NET
TAS-303	50.9 ± 1.9	1560 ± 77	1290 ± 69	31	253
duloxetine	3.42 ± 0.25	0.283 ± 0.033	157 ± 7	0.08	46

The affinity of each compound was measured by inhibition of [^{125}I]RTI-55 binding for NET and DAT, and [^{3}H]paroxetine binding for SERT to respective membranes. Ki values for receptor binding were determined from three independent experiments using 7 drug concentrations.

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Table 2. Inhibition of monoamine uptake *in vitro* by TAS-303 and duloxetine

Compound	Monoamine Uptake IC_{50} ± S.E.M. (nM)			Selectivity	
	NET	SERT	DAT	SERT/NET	DAT/NET
TAS-303	38.9 ± 2.8	3080 ± 240	28300 ± 2000	79	726
duloxetine	4.03 ± 0.28	3.52 ± 0.56	149 ± 9	0.87	37

IC_{50} values for monoamine uptake were determined from three independent experiments using 7 drug concentrations.

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Table 3. Effect of vehicle, TAS-303, or duloxetine on NE-induced contraction of rat isolated urethral strips.

Compound	Concentration (nM)	EC_{50} (log_{10} mol/L)			
Vehicle	-	-4.49	±	0.09	-
	1st vehicle	-4.54	±	0.11	n.s.
	2nd vehicle	-4.57	±	0.08	n.s.
	3rd vehicle	-4.62	±	0.07	n.s.
	4th vehicle	-4.64	±	0.04	n.s.
	5th vehicle	-4.63	±	0.08	n.s.
Duloxetine	-	-4.40	±	0.08	-
	10	-4.43	±	0.08	*
	30	-4.57	±	0.06	**
	100	-4.80	±	0.02	**
	300	-5.00	±	0.08	**
	1000	-5.12	±	0.04	**
TAS-303	-	-4.35	±	0.10	-
	10	-4.48	±	0.12	n.s.
	30	-4.69	±	0.08	**
	100	-4.77	±	0.08	**
	300	-4.95	±	0.08	**
	1000	-5.12	±	0.11	**

EC_{50} of NE-induced contractions was obtained from a sigmoidal curve under variable E_{max} conditions. Results are shown as mean ± S.E.M. (n = 5 for each compound in each tissue).

n.s. not significant; $P > 0.05$ vs. control (pre-treatment).

*, **; $P < 0.05$, 0.01 vs. control (pre-treatment) by a paired student's *t*-test.

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Table 4. Pharmacokinetic parameters after single oral administration of TAS-303 at 1, 3, or 10 mg/kg in normal female rats.

Dose (mg/kg)	T_{max} (hr)	C_{max} (nM)
1	0.75 ± 0.29	11.7 ± 5.0
3	0.75 ± 0.29	49.1 ± 13.9
10	1.25 ± 0.50	453.0 ± 28.8

T_{max}, time to peak concentration; C_{max}, maximum plasma concentration.

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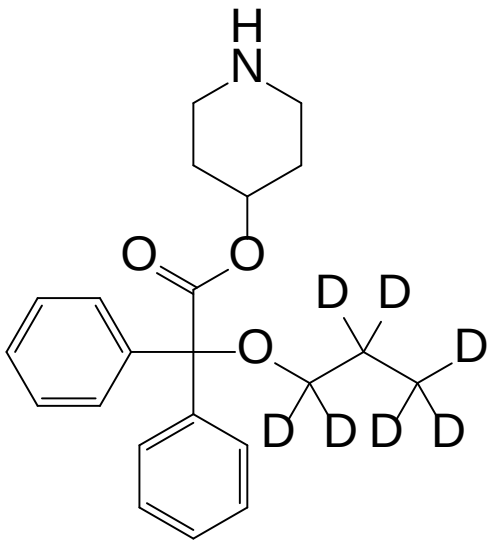

Figure 1

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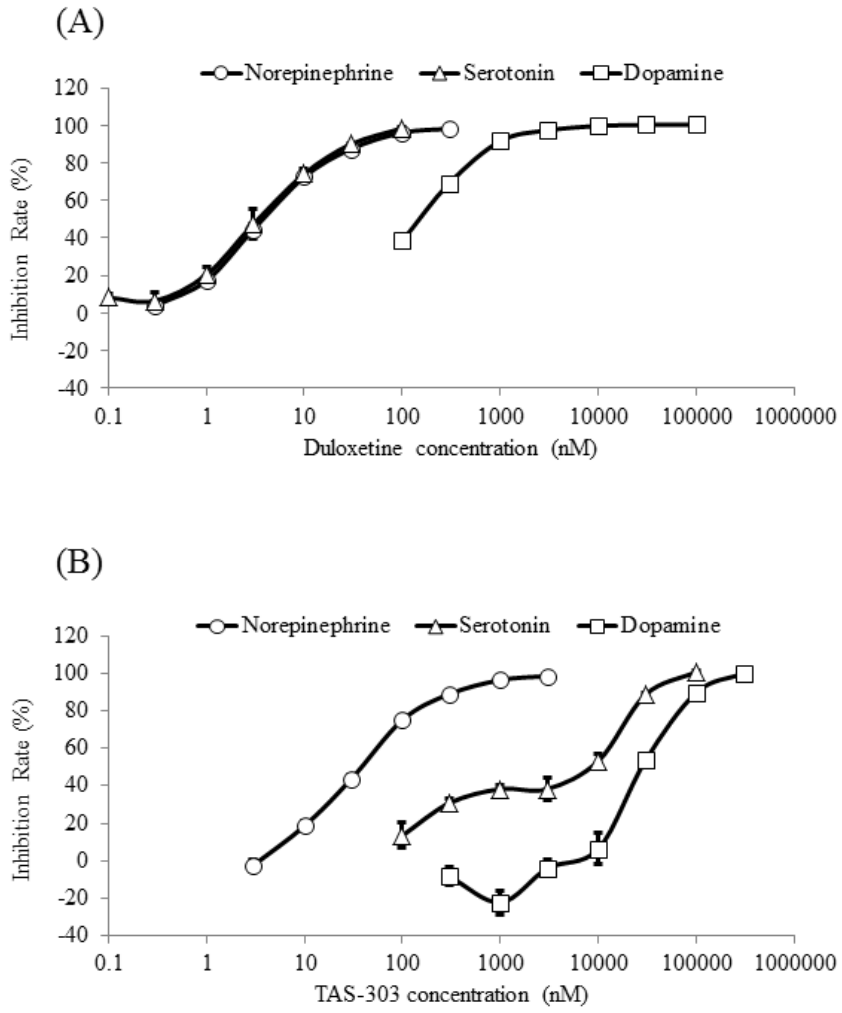

Figure 2

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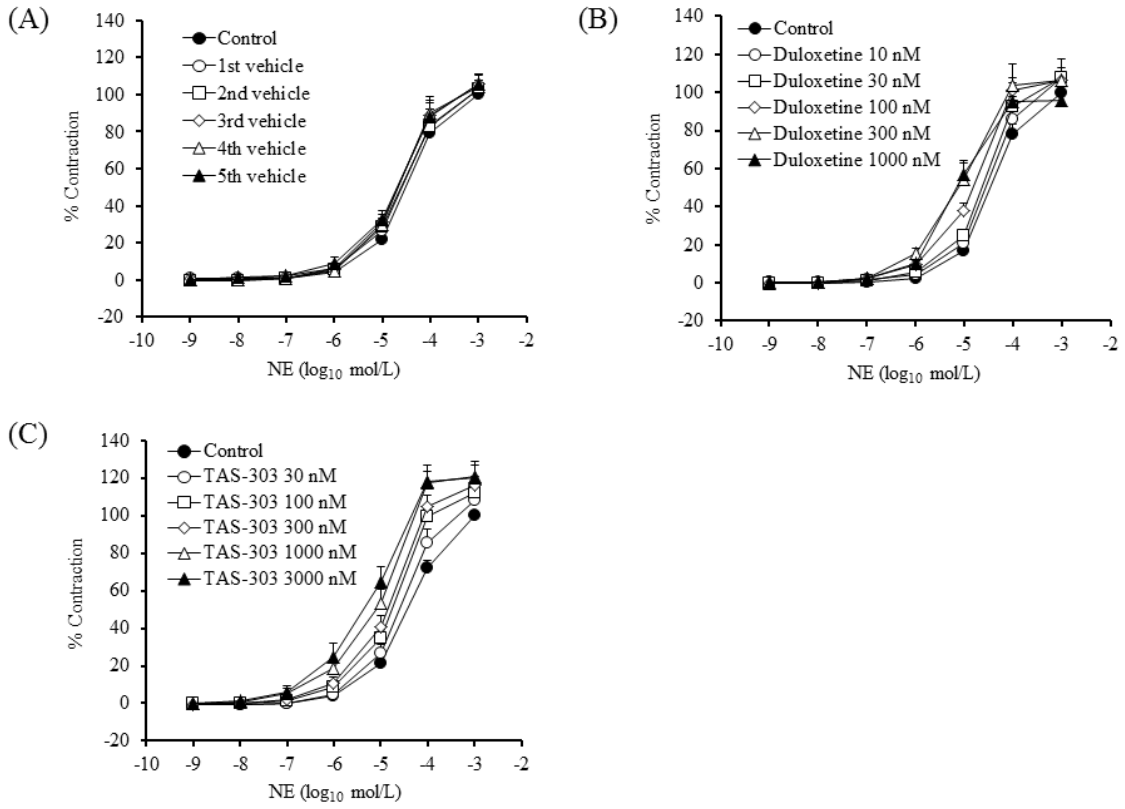

Figure 3

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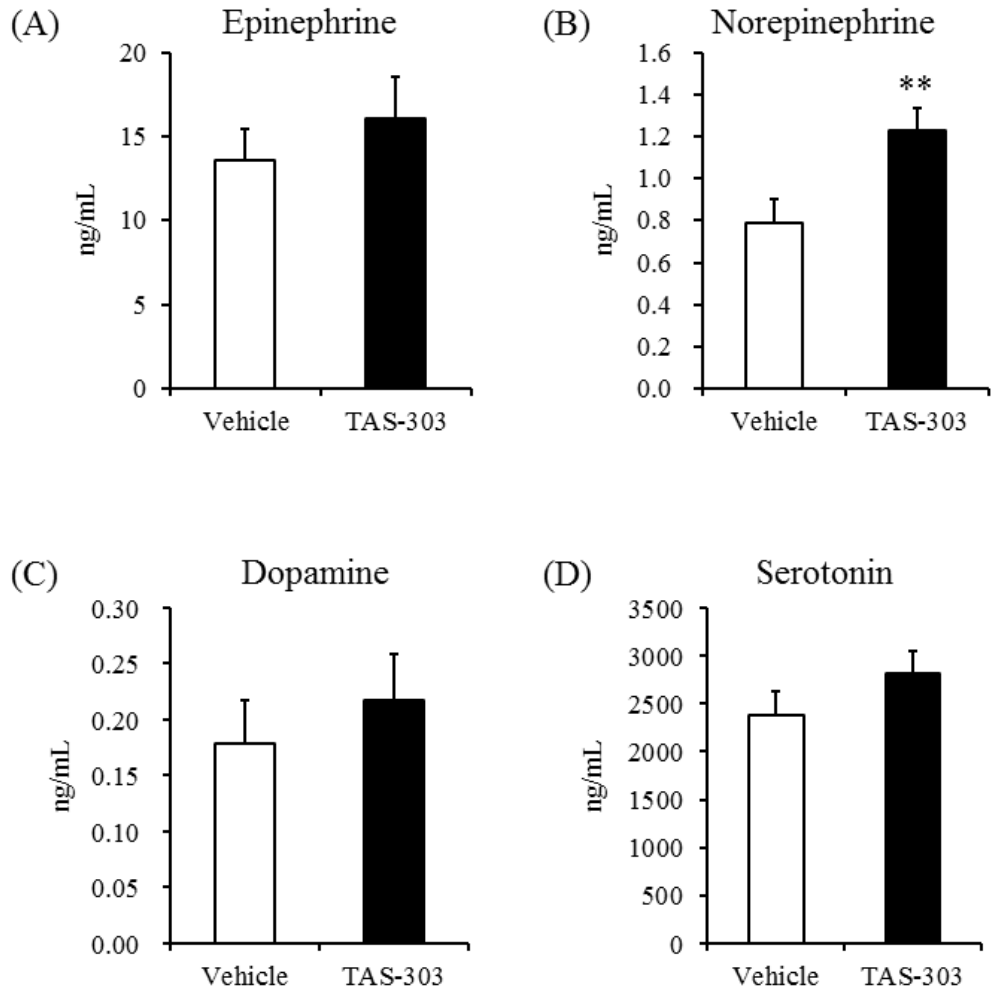

Figure 4

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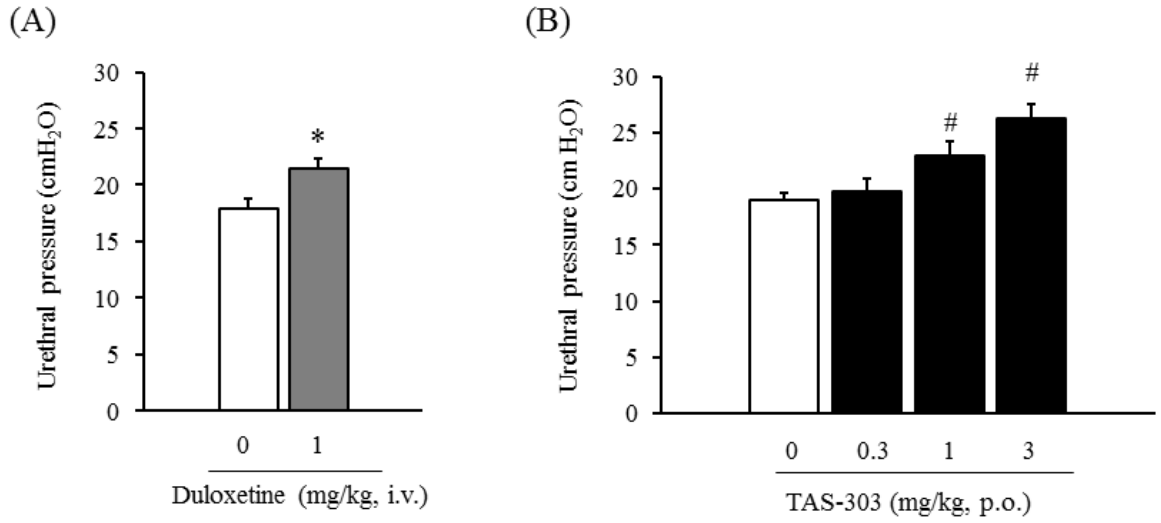

Figure 5

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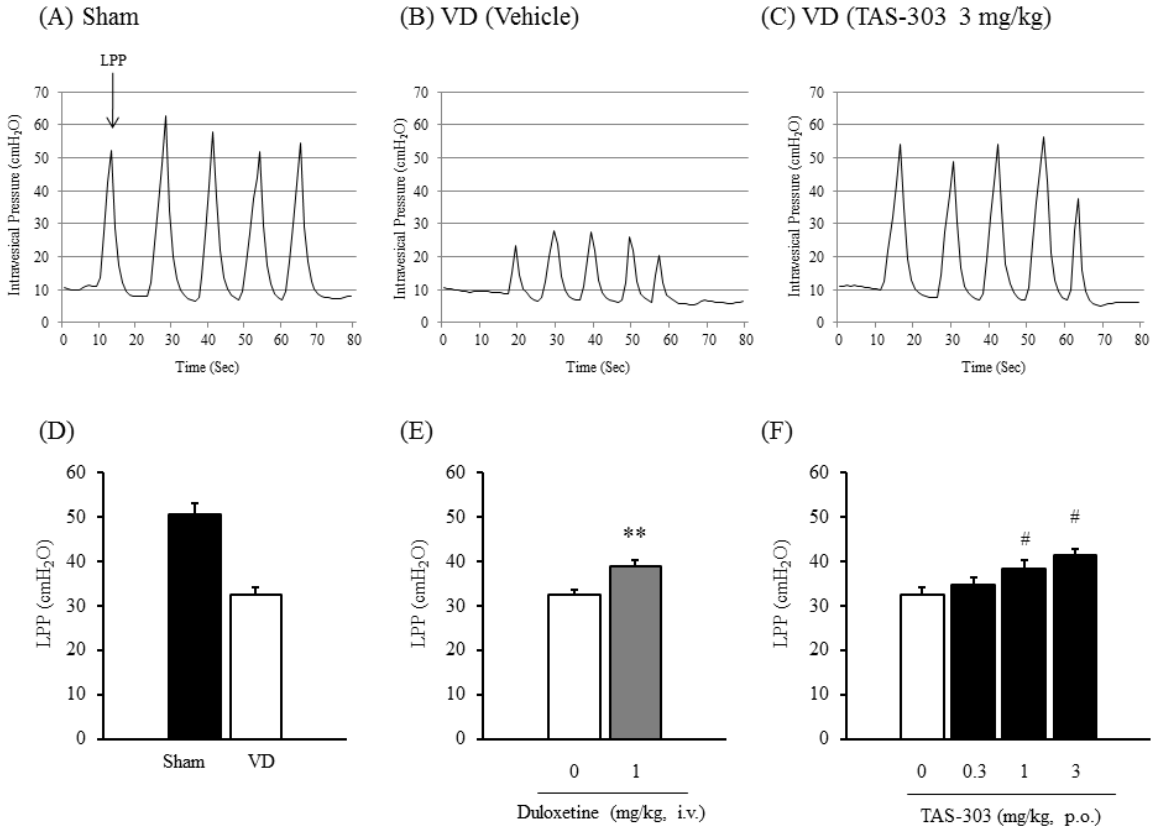

Figure 6

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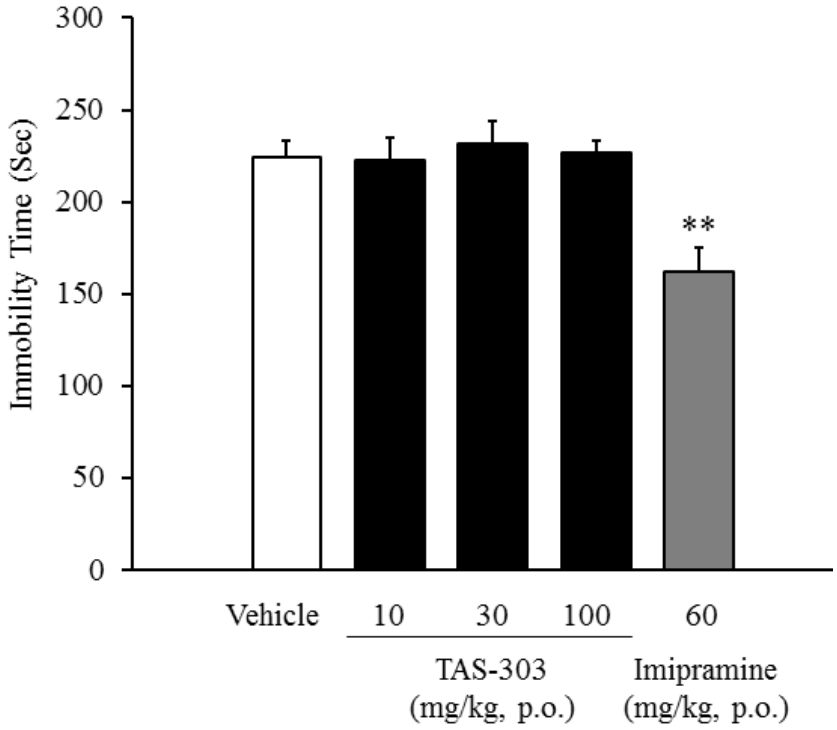

Figure 7