Increasing Effects of Selective 5-Hydroxytryptamine Type 2C Receptor Stimulation on Evoked Momentary Urethral Closure in Female Rats and Humans

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Received March 23, 2021; Accepted April 5, 2021

ABSTRACT

Under healthy conditions, more than one urethra-closing reflex, including both bladder afferent–independent and −dependent actions, function during momentary elevation of intravesical (bladder) pressure to prevent urinary incontinence. In the current study, the effects of a novel selective 5-hydroxytryptamine type 2C (5-HT2C) receptor agonist, TAK-233, on evoked momentary urethral-closing functions were investigated in female rats and humans to elucidate 5-HT2C receptor functions. In anesthetized female rats, TAK-233 dose-dependently and significantly increased urethral resistance during sneezing in rats with distended vaginas and bilaterally transected pelvic nerves. The drug also dose-dependently and significantly increased urethral resistance during momentary intravesical pressure elevation by electrical stimulation of abdominal muscles in rats with a transected spinal cord at the T8–T9 level and intact pelvic nerves. The increased effects observed during electrical stimulation were abolished by either an intravenously administered selective 5-HT2C receptor antagonist, SB 242084, or bilateral transection of the pelvic nerves or somatic nerves innervating the external urethral sphincter and pelvic floor muscles. In the spinal cord–transected and pelvic nerve–intact rats, TAK-233 enlarged the urethral-closing responses induced by both passive and abrupt intravesical pressure elevation, measured by a microtip transducer located in the middle urethra. Additionally, the effects of TAK-233 on the stimulus threshold of urethral contractile responses induced by transcranial magnetic stimulation were investigated in healthy female volunteers. The drug dose-dependently and significantly lowered this stimulus threshold, indicating an increased sensitivity of the response. These results demonstrate that 5-HT2C receptor stimulation enhances the evoked momentary urethra-closing functions in both female rats and humans.

SIGNIFICANCE STATEMENT

5-hydroxytryptamine (serotonin) type 2C (5-HT2C) receptor stimulation by TAK-233 enhanced urethral resistance in rats during an evoked momentary event in which the bladder afferent–independent or −dependent reflex functions via striated muscle–mediated mechanisms. The increases in sensitivity of transcranial magnetic stimulation–evoked urethral contractile responses in healthy female subjects indicates that this mechanism also functions in humans. The evoked momentary conditions activating these reflexes provide a suitable model to demonstrate the effects of 5-HT2C receptor stimulation.

Introduction

Stress urinary incontinence generally occurs as a result of defects in various passive and reflex mechanisms that maintain urethral closure in the presence of elevated abdominal pressure (Yoshimura and Miyazato, 2012). With respect to nerve-mediated mechanisms, at least two urethra-closing reflex mechanisms function during the abrupt and momentary elevation of intravesical (bladder) pressure (Pves) (Kamo et al., 2009, Yoshimura and Miyazato, 2012). One is a bladder afferent–dependent reflex where both abrupt and passive Pves elevation induces urethra-closing responses in spinal cord–transected rats (spinal reflex) and where the responses are totally abolished by the bilateral transection of the pelvic nerves containing bladder afferent nerves (Kamo et al., 2004). A 1-second electrical stimulation of abdominal muscles induces momentary passive Pves elevation in rats, and bilateral transection of the pelvic nerves greatly reduces urethral resistance during

The data described in Figs. 2–6 and in the subsection “Reflex Urethra-Closing Responses in Rats” were previously published in the International Publication W02019/131902A1 after filing international patent application (Kamo I, Mueller-Flock N, Takeda Pharmaceutical Company Ltd. Therapeutic agent for stress urinary incontinence and fecal incontinence. 04 July 2019).

This work was supported by Takeda Pharmaceutical Company Ltd. This work received no external funding.

https://dx.doi.org/jpet.121.000573

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; 5-HT2A, 5-hydroxytryptamine type 2A; 5-HT2B, 5-hydroxytryptamine type 2B; 5-HT2C, 5-hydroxytryptamine type 2C; LPP, leak point pressure; MT, motor threshold; Pves, intravesical pressure; S-LPP, sneeze leak point pressure; TMS, transcranial magnetic stimulation.

https://dx.doi.org/jpet.121.000573

J Pharmacol Exp Ther 378:60–68, August 2021
electrical stimulation (Kamo and Hashimoto, 2007), demonstrating that the bladder afferent–dependent spinal reflex contributes to the prevention of urinary incontinence during passive $P_{\text{ves}}$ elevation. The other reflex is a bladder afferent–dependent mechanism and functions during sneezing in spinal cord–intact rats (Kamo et al., 2003, 2006 and 2009). Clear middle urethra–closing responses are observed during sneezing, and those responses are greatly reduced after bilateral transection of the somatic nerves innervating the external urethral sphincter and pelvic floor muscles, whereas bilateral transection of both pelvic and hypogastric nerves had no effect (Kamo et al., 2003), demonstrating that the reflex is bladder afferent–independent. Bilateral transection of the somatic nerves induces a clear reduction in urethral resistance during sneezing in rats (Kamo et al., 2003), indicating that this reflex contributes to the prevention of urinary incontinence during sneezing. In addition, the urethral reflex is considered to be one component of the preprogrammed sneeze reflexes observed throughout the body, since urethral responses are observed before the start of $P_{\text{ves}}$ elevation during sneezing (Kamo et al., 2003). Since a sneeze is a very brief and sudden event lasting for up to 0.15 second in rats (Kamo et al., 2003), the passive $P_{\text{ves}}$ elevation–induced urethral reflex (bladder afferent–dependent spinal reflex) may occur too late to prevent any urine leakage from the urethral orifice (Kamo et al., 2009). These findings indicate that at least two urethra-closing reflexes are essential to prevent urinary leakage during various events that elevate $P_{\text{ves}}$, therefore it is considered important to identify relevant mechanisms that enhance both types of reflexes when developing treatments for stress urinary incontinence.

5-hydroxytryptamine (serotonin; 5-HT) receptors are divided into at least 14 different receptor subtypes that have been classified into seven major families (Hoyer et al., 2002). Stimulation of 5-HT type 2C (5-HT2C) receptor increased the urinary storage function of the urethra in a pioneering study in guinea pigs (Conlon et al., 2012). In rats, receptor stimulation increased the bladder afferent–independent urethra-closing reflex during sneezing (Miyazato et al., 2009; Suzuki et al., 2018; Ouchi et al., 2018). A 5-HT type 2B (5-HT2B)/5-HT2C agonist, meta-chlorophenylpiperazine, and a 5-HT2C agonist, CP-809101, enhanced the urethra-closing responses during sneezing in rats, and these responses were totally abolished by RS-102221 and SB 242084, selective 5-HT2C receptor antagonists, respectively. However, the effect on urethral functions via the bladder afferent–dependent spinal reflex remains unclear.

In the current study, to clarify the effects of 5-HT2C receptor stimulation on urethral function, TAK-233, another selective 5-HT2C receptor agonist, was investigated for effects on the urethra-closing reflex under both the bladder afferent–dependent and –independent mechanisms. Furthermore, to better understand these effects in humans, the effects of TAK-233 on transcranial magnetic stimulation (TMS)–induced urethra-closing responses were investigated in healthy female subjects.

**Materials and Methods**

**Nonclinical Studies**

**Drugs.** N-methyl-N-(1-methylethyl)-6,7,8,9-tetrahydropropazine[2,3-f][1,4]oxazepin-3-amine monohydrochloride (TAK-233) (Fig. 1; Example 8 in the patent WO2010/147226A1 [Sasaki S, Kusumoto T, Nomura I, and Maezaki H. Takeda Pharmaceutical Company Ltd. Pyrazinoxazine derivatives. 23 December 2010]) and lorcaserin hydrochloride (lorcaserin) were synthesized by Takeda Pharmaceutical Company Ltd. 5-HT, duloxetine hydrochloride (duloxetine), and SB 242084 were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Kem- protec (Middlesbrough, UK), and Tocris Bioscience (Bristol, UK), respectively. Drugs were suspended in 0.5% methylcellulose (Shin-Etsu Chemical CO., Ltd., Tokyo, Japan) solution for intraduodenal administration and dissolved in $N,N$-dimethylacetamide-polyethylene glycol 400 (1:1) for intravenous administration. Drug doses are listed as the weight of the free base.

In addition, the urethra-closing responses are essential to prevent urinary leakage during various events that elevate $P_{\text{ves}}$, therefore it is considered important to identify relevant mechanisms that enhance both types of reflexes when developing treatments for stress urinary incontinence.

**In Vitro Receptor Agonist Potency.** Recombinant human 5-HT2C receptor–expressing CHO-K1 cells purchased from Euroscreen (Gosselies, Belgium) were cultured in supplemented UltraCHO media, and recombinant human 5-HT type 2A (5-HT2A), human 5-HT2B, or rat 5-HT2C receptor–expressing CHO/dhFR- cells generated by Takeda Pharmaceutical Company Ltd. were cultured in supplemented alpha Modified Eagle Minimum Essential Medium.

5-HT receptor agonist potency of 5-HT, TAK-233, and lorcaserin was assessed by performing calcium influx assays using the integrated detection and liquid handling robotics, CellLux (PerkinElmer, Waltham, MA). The recombinant receptor-expressing cells were seeded into black-walled clear-bottom 384-well plates at a density of 10,000 cells per well, treated with 10 mM sodium butyrate, and grown for approximately 24 hours at 37 °C in a 5% CO2 incubator. The culture media were removed, and the cells were loaded with 40 μM/well of Ca2+ indicator dye solution, Ca Screening Kit Fluo 4 (DOJINDO laboratories, Kumamoto, Japan), for around 60 minutes at 37 °C in a 5% CO2 incubator. The plates were left for approximately 10 minutes at room temperature and placed into the CellLux to measure intracellular Ca2+ mobilization. Fluorescence was monitored at a 2-second interval for 30 seconds. After 10-second basal signal recording, 20 μM of 3-fold compound solution was added by CellLux-equipped 96-well pipettor. CellLux repeated the sequence four times.

The collected fluorescence counts were calculated by subtracting the background fluorescence in the same well. The change in fluorescence counts for each compound was determined by subtracting the minimum fluorescence count from maximum fluorescence count in the same well. The change in fluorescence counts was normalized to the response observed with 10 μM of 5-HT. The values of EC50 were estimated by a two-parameter logistic regression analysis. The relative potency was calculated by dividing EC50 value of a compound by EC50 value of 5-HT. The selectivity for human 5-HT2C receptors was calculated by dividing the relative potency against human 5-HT2A or human 5-HT2B receptors by the relative potency against human 5-HT2C receptor.

**Sneeze Leak Point Pressure.** The experiment was conducted based on the method described in literature (Kamo et al., 2003). Rats were anesthetized with isoflurane (Abbott Japan, Tokyo, Japan). An 8-Fr Foley balloon catheter (TOP Corporation, Tokyo, Japan) was inserted into the vagina, inflated with 3 ml water for 2 hours, and...
removed. Four days after the vaginal distention, rats were anesthetized with urethane (Wako Pure Chemical Industries) and isoflurane. The bladder and the duodenum were exposed through an abdominal incision. PE-50 and PE-100 polyethylene catheters (Intramedic, Becton Dickinson and Company, Franklin Lakes, NJ) were inserted into the duodenum and the bladder, respectively, and were secured with ligatures. A 3.5-Fr catheter with a side-mounted SPR-524 microtip transducer (Millar Instruments, Houston, TX) connected to an amplifier (ML117, ADInstruments, Castle Hill, NSW, Australia) was inserted into the bladder. The pelvic nerves were cut bilaterally to prevent reflex bladder contractions. The abdominal incision was closed with sutures. 

Sneeze leak point pressure (S-LPP) expressing urethral resistance during sneezing was measured as follows. After the bladder was emptied, 0.3 ml of 0.1% Evans blue (Wako Pure Chemical Industries)–saline solution was injected into the bladder. A fiber of brush was inserted into the nostril to induce sneeze reflexes while recording changes in \( P_{\text{ves}} \) at a sampling rate of 1000 Hz while examining if leakage of the Evans blue solution was observed from the urethral orifice. \( P_{\text{ves}} \) was recorded using data acquisition software (Chart, ADInstruments) on a computer system equipped with a PowerLab analog-to-digital converter (ADInstruments). The maximal \( P_{\text{ves}} \) during each sneeze event was evaluated, and the lowest pressure that induced fluid leakage was defined as S-LPP.

### Leak Point Pressure During Electrical Stimulation of Abdominal Muscles

The experiments were conducted based on the methods described in literature (Kamo and Hashimoto, 2007). Rats were anesthetized by intraperitoneal administration of urethane, and isoflurane inhalation was added during surgery, if necessary. Their spinal cord was transected at the T8–T9 level after lamincotomy to prevent reflex bladder contractions. After the abdomen was opened, nerves innervating the lower urinary tracts and the pelvic floor muscles were transected as indicated in results. PE-100 and PE-50 catheters were inserted into the bladder and the duodenum, respectively, and secured. The abdomen was then closed with sutures. The bladder catheter was connected to a pressure transducer (Life kit; Nihon Kohden, Tokyo, Japan) and an amplifier (AP-641G; Nihon Kohden). Two sites of abdominal skin were cut, and the stimulus needle electrodes were inserted in exposed abdominal muscles. After the bladder was emptied, 0.3 ml of 0.1% Evans blue–saline solution was injected into it. While the \( P_{\text{ves}} \) was digitally recorded at a sampling rate of 500 Hz using data acquisition software (Acknowledge; BIOPACK Systems), the exposed abdominal muscles were stimulated with rectangular electric pulses (50 Hz, 0.5-millisecond width pulse trains lasting 1 second) by an electrical stimulator (SEN-34301; Nihon Kohden) and an isolator (SS-202-J; Nihon Kohden). The stimulus intensity was gradually increased from 1 V to 10 V to increase the \( P_{\text{ves}} \) step by step, and the lowest \( P_{\text{ves}} \) at which fluid leakage from the urethral orifice was observed was regarded as leak point pressure (LPP) indicating urethral resistance.

### Reflex Urethra-Closing Responses

The experiments were conducted based on the method described in literature (Kamo and Hashimoto, 2007). The spinal cord of urethane-anesthetized rats was transected at the T8–T9 level after lamincotomy. The bladder was exposed, and a PE-100 catheter was inserted into it. The bladder neck was ligated with a suture to prevent fluid leakage from the bladder into the urethra. \( P_{\text{ves}} \) was controlled by connecting a bladder catheter to a saline reservoir and a pressure transducer (MLT0670, AD Instruments) via three-way stopcocks. A SPR-524 microtip transducer catheter was inserted into the middle urethra from the urethral orifice. The microtip transducer catheter and the pressure transducer were connected to an amplifier (ML117, AD Instruments), and urethral responses were digitally recorded at a sampling rate of 1000 Hz using data acquisition software (Chart, AD Instruments) on a computer system equipped with an analog-to-digital converter (PowerLab4/52, AD Instruments). \( P_{\text{ves}} \) was abruptly increased by elevating the reservoir and maintaining \( P_{\text{ves}} \) for 30 seconds at 50 cmH₂O while the urethral response was recorded. The average values measured with the microtip transducer for 30 seconds before and during increment of \( P_{\text{ves}} \) were evaluated, and the difference of the average values was defined as a urethra-closing response.

#### Statistical Analysis

Data are means ± S.E.M. Data are analyzed with Dunnett’s test, Student’s \( t \) test, or Welch’s test, and \( P \) values less than 0.05 were considered to be significant.

### Clinical Study: A Randomized, Double-Blind, Single-Dose, 4 × 4 Crossover Pharmacodynamic Study

This study was approved by the Institutional Review Board at the study site and was conducted in accordance with the ethical principles of the Declaration of Helsinki, the International Conference on Harmonization E6 Guidelines for Good Clinical Practice, and all applicable local laws and regulations.

Healthy adult Japanese women from 20 to 40 years of age, weighing at least 45 kg, with a body mass index between 18.5 kg/m² and 25.0 kg/m², who had signed and dated a written informed consent, and who met inclusion criteria were randomized into four administration sequences. Main inclusion criteria were as follows:

1. In the opinion of the investigator or subinvestigator, a subject who was capable of understanding and complying with the protocol requirements.
2. A female subject who did not have any conditions that could affect the urethra function (including no vaginal delivery or urethral surgery, etc.).

A subject received a single dose of either TAK-233 (Takeda Pharmaceutical Company Ltd.) 20 mg, TAK-233 90 mg, duloxetine (Eli Lilly Japan, Kobe, Japan) 40 mg, or placebo (both TAK-233 placebo solution and duloxetine placebo capsule) in periods 1 through 4 in accordance with the predefined treatment in each administration sequence. The study consisted of the screening period (day –28 to day –2), treatment period (day –1 to day 2 in periods 1 through 4), and follow-up examination (day 8 in period 4). TAK-233, duloxetine, and placebo were administered in the fasted state on day 1 in each period. All subjects who underwent the pharmacodynamic tests on day 1 received 500 mg of levofloxacin (CRAVIT; Daiichi-Sankyo, Tokyo, Japan) after the test to avoid urinary tract infection. The washout interval between the two consecutive periods was at least 7 days.

Based on the method described in the literature (Boy et al., 2006), the pharmacodynamic test identified a motor threshold (MT) for inducing urethral sphincter contraction in response to TMS as a primary endpoint and measured urethral pressure profile at rest as a secondary endpoint. Briefly, after bladder emptying, a 110-mm double cone coil (Magstim, Whittland, UK) that was designed to fit overhead and connected to a Magstim Rapid stimulator (Magstim) was positioned near the vertex for TMS, and a dual microtip pressure transducer catheter (Unisensor AG, Attikon, Switzerland) having its transducers lateral in the three o’clock position was positioned in the bladder and the urethra by inserting it from the urethral orifice. After identifying maximal tolerable TMS within maximal stimulator output and suitable catheter position in the urethra for observing urethral responses, TMSs with decreasing intensities by 5% of the maximal stimulator output were delivered, and the minimum intensity that produced a urethral contractile response was determined as MT. Urethral pressure profiles at rest were recorded during pulling the catheter at 1 mm/s speed, and the maximal pressure was measured. The pharmacodynamic tests were performed once before treatment and three times post-treatment on day 1.

Blood samples were collected, and plasma drug concentrations were measured by a method with liquid chromatography with tandem mass spectrometry.

Results are expressed as the means ± S.D., and statistical differences among groups were analyzed by ANOVA for the primary endpoint.

Safety was also assessed as an endpoint. Treatment-emergent adverse events were coded using the Medical Dictionary for Regulatory Affairs (MedDRA).
Activities version 17.1., and vital signs, weight, ECGs, and clinical laboratory test values were also measured.

Results

In Vitro Receptor Agonist Potency

5-HT, TAK-233, and lorcaserin showed agonist activities on human and rat 5-HT\textsubscript{2C} receptors, with relative agonist potencies of TAK-233 and lorcaserin to 5-HT being slightly weaker on rat 5-HT\textsubscript{2C} receptors compared with those on human 5-HT\textsubscript{2C} receptors (Table 1). Selectivity of TAK-233 and lorcaserin for human 5-HT\textsubscript{2C} receptors over human 5-HT\textsubscript{2A} receptors was 26-fold and 11-fold (TAK-233 and lorcaserin, respectively), and selectivity over 5-HT\textsubscript{2B} receptors was 282-fold and 70-fold (TAK-233 and lorcaserin, respectively), indicating that selectivity of TAK-233 is slightly higher compared with that of lorcaserin, a selective 5-HT\textsubscript{2C} receptor agonist marketed for the treatment of obesity.

In a broad screen for receptor binding affinity and effects on various ion channel and enzyme activities, other than the 5-HT\textsubscript{1B} and nonselective sigma receptors (50\% and 54\% radioligand binding inhibition at 10\textsuperscript{m}, respectively).

S-LPP

In rats without vaginal distension, two out of four rats did not show fluid leakage from the urethral orifice during sneezing, whereas in all rats with vaginal distension, fluid leakage was observed during sneezing with an S-LPP of 47.7 ± 2.1 cmH\textsubscript{2}O.

TAK-233 at 1 and 3 mg/kg dose-dependently and significantly increased the S-LPP (Fig. 2). Duloxetine significantly increased the S-LPP at all tested doses; however, the maximal effect of TAK-233 was significantly higher than that of duloxetine (Fig. 2).

LPP During Electrical Stimulation of Abdominal Muscles

All rats with unilateral transection of the nerves to ilioococygeus/pubococcygeus muscles showed fluid leakage from the urethral orifice by electrical stimulation. TAK-233 dose-dependently and significantly increased LPPs with an effect at 3 mg/kg intraduodenally of approximately 30 cmH\textsubscript{2}O (Fig. 3). Duloxetine also dose-dependently and significantly increased the LPP with an effect at 20 mg/kg intraduodenally of approximately 10 cmH\textsubscript{2}O (Fig. 3). In a preliminary experiment, the duloxetine effects at 20 mg/kg intraduodenally were maximal among the tested doses up to 40 mg/kg intraduodenally.

The urethral resistance-increasing effects of TAK-233 at 3 mg/kg intraduodenally were dose-dependently inhibited by SB 242084, a specific 5-HT\textsubscript{2C} receptor antagonist (Kennett et al., 1997; Fig. 4).

In rats with intact nerves innervating the lower urinarytracts and pelvic floor muscles, TAK-233 significantly increased the LPP with the effects totally abolished by bilateral transection of the pelvic nerves (Table 2). Although bilateral transection of the hypogastric nerves innervating urethral smooth muscles did not affect TAK-233’s effects, bilateral transection of both the pudendal nerves innervating the external urethral sphincter and the coccygeus muscle and the nerves to ilioococygeus/pubococcygeus muscles greatly inhibited these effects (Table 2), indicating a greater contribution of the striated muscle component to the effects of TAK-233.

Reflex Urethra-Closing Responses in Rats

Abrupt elevation of P\textsubscript{ves} from 0 cmH\textsubscript{2}O to 50 cmH\textsubscript{2}O induced reflex urethra-closing responses (Fig. 5; Kamo and Hashimoto., 2007). The responses were measured two times before and 30 minutes after drug administration, respectively, and the ratio of post-value to pre-value was calculated with the means of the two trials. TAK-233 at 3 mg/kg intraduodenally significantly increased the responses with a post/pre ratio of 2.82 ± 0.29 (mean ± S.E.M. of 8 rats; P < 0.001 vs. vehicle-treated group by Welch’s test), whereas the vehicle had no effect with a post/pre ratio of 1.11 ± 0.13 (mean ± S.E.M. of 8 rats). In a separate experiment, duloxetine at 20 mg/kg intraduodenally also significantly enlarged the responses with a post/pre ratio of 2.00 ± 0.28 (mean ± S.E.M. of 8 rats; P < 0.05 vs. vehicle-treated group by Welch’s test), whereas the vehicle had

Table 1

<table>
<thead>
<tr>
<th>Receptor</th>
<th>5-HT</th>
<th>TAK-233</th>
<th>Lorcaserin</th>
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<td>Human 5-HT\textsubscript{2C}</td>
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<td>61 (36–104)</td>
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<td>Response at 10 \textmu M (%)</td>
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CI, confidence interval.

*Agonistic response at 10 \textmu M was normalized to that observed with 5-HT at 10 \textmu M.

*Relative potency was calculated by dividing EC\textsubscript{50} value of a compound by EC\textsubscript{50} value of 5-HT.
no effect with a post/pre ratio of 1.07 ± 0.11 (mean ± S.E.M. of 8 rats).

A Randomized, Double-Blind, Single-Dose, 4 × 4 Crossover Pharmacodynamic Study

Effects of TAK-233 on urethra-closing functions in healthy female subjects were examined. The mean subject age was 22.4 years (S.D., 2.04), the mean weight was 50.91 kg (S.D., 3.346), and the mean body mass index was 19.57 kg/m² (S.D., 0.929). Demographic and other baseline characteristics were well balanced across the administration sequences. Twenty-four eligible subjects at one study site in Japan received the study drug. Of the eligible subjects, 23 subjects completed the study; one subject withdrew due to an adverse event that was considered unrelated to the study drug. Each of the 24 eligible subjects was allocated to a four-part administration sequence (six subjects/sequence).

The mean MT for urethral sphincter contraction in response to TMS was significantly decreased by TAK-233 at 20 mg and 90 mg and duloxetine at 40 mg with no change after placebo treatment (Fig. 6). The maximum MT change from baseline was −8.1%, −17.2%, and −11.8% (TAK-233 20 mg at 0.5 hour, TAK-233 90 mg at 3 hours, and duloxetine 40 mg at 6 hours, respectively), indicating a greater change from baseline after TAK-233 at 90 mg compared with the other treatment groups (Fig. 6).

In the TAK-233 90 mg group, the mean change from baseline in the maximum urethral pressure at rest along the entire functional length of the urethra increased at 0.5 hour after dose followed by decreases at later time points (Fig. 7). In the duloxetine 40 mg group, the mean change from baseline increased at 3 hours and 6 hours after dose (Fig. 7). In the TAK-233 20 mg group and placebo groups, the mean change from baseline appeared unaffected.

TAK-233 was rapidly absorbed after a single oral administration, and the maximal plasma level was observed at 0.5 hour and 2 hours (20 mg and 90 mg, respectively; Table 3). The maximal plasma level of duloxetine was observed at 6 hours after administration (Table 3).

No deaths or serious adverse events and no obvious changes in mean values of clinical laboratory tests were reported during this study.

Discussion

Although many events in daily activities cause a rise in abdominal pressure with bladder compression, nerve-mediated active urethral closure contributes to the prevention of urinary incontinence under normal conditions (Yoshimura and Miyazato, 2012). Rat studies investigating urethral resistance and reflex urethral responses demonstrate...

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**Fig. 2.** Effects of TAK-233 and duloxetine on the urethral resistance during sneezing in rats with vaginal distention. In all rats, pelvic nerves were bilaterally transected. S-LPPs were measured before and 30 minutes after intraduodenal (i.d.) drug administration. Data are expressed as means ± S.E.M. of increase in S-LPP. Number of rats evaluated were 8, 8, 4, 6, 8 and 3 (vehicle, TAK-233 1 and 3 mg/kg, duloxetine 10, 20, and 40 mg/kg, respectively). **P < 0.01, ***P < 0.001 compared with the vehicle-treated group by Dunnett’s test. ###P < 0.001 compared by Student’s t test.

**Fig. 3.** Effects of TAK-233 (A) and duloxetine (B) on the urethral resistance during momentary intravesical pressure elevation by electrical stimulation of abdominal muscles in rats. In all rats, the spinal cord was transected at the T8–T9 level, and the nerve to iliococcygeus/pubococcygeus muscles was unilaterally transected to reduce urethral resistance. LPPs were measured before and 30 minutes after intraduodenal (i.d.) drug administration. Data are expressed as means ± S.E.M. in 6 or 8 rats for the experiment with TAK-233 (A) or duloxetine (B), respectively. Means ± S.E.M. of baseline LPP values were 46.1 ± 6.4 cmH₂O, 47.2 ± 5.7 cmH₂O, 47.3 ± 5.0 cmH₂O, and 47.6 ± 3.1 cmH₂O (TAK-233 0, 0.3, 1, and 3 mg/kg intraduodenally, respectively) and 35.6 ± 4.3 cmH₂O, 36.6 ± 3.0 cmH₂O, 35.3 ± 3.0 cmH₂O, and 36.9 ± 2.8 cmH₂O (duloxetine 0, 5, 10, and 20 mg/kg, intraduodenally, respectively). *P < 0.05, **P < 0.01, ***P < 0.001 compared with the vehicle-treated group by Dunnett’s test.
that at least two kinds of reflexes function during this rise in $P_{\text{ves}}$ (Kamo et al., 2009; Yoshimura and Miyazato, 2012). One is the bladder afferent–dependent spinal reflex induced by passive $P_{\text{ves}}$ elevation, and the other is the bladder afferent–independent reflex. The latter reflex functions as a preprogrammed reflex at least during sneezing, and 5-HT$_{2C}$ receptor stimulation by meta-chlorophenylpiperazine or CP-809101 increases the sneeze-induced urethral response under different conditions (Miyazato et al., 2009, Suzuki et al., 2018). In the current study, another specific 5-HT$_{2C}$ receptor agonist, TAK-233, also increased urethral resistance during sneezing in female rats. These findings obtained across a range of experimental conditions with different drugs demonstrate that 5-HT$_{2C}$ receptor stimulation increases urethral closure during sneezing in rats via an enhancement of the bladder afferent–independent reflex.

The effects of TAK-233 on passive $P_{\text{ves}}$ elevation–induced urethra-closing responses were investigated to determine if 5-HT$_{2C}$ receptor stimulation enhances the bladder afferent–dependent spinal reflex. TAK-233 significantly increased the evoked responses, indicating enhancement of the spinal reflex. TAK-233 also increased the urethral resistance in spinal cord–transected rats during electrical stimulation of abdominal muscle–induced passive $P_{\text{ves}}$ elevation. The increased effects were not observed after bilateral pelvic nerve transection that would have totally abolished the $P_{\text{ves}}$ elevation–induced reflex urethral contraction itself (Kamo et al., 2004). These findings demonstrate that 5-HT$_{2C}$ receptor stimulation also enhances the bladder afferent–dependent spinal reflex to elevate urethral resistance during passive $P_{\text{ves}}$ elevation in rats.

The effects of TAK-233 on the urethral resistance during sneezing in rats were significantly larger than those of duloxetine, a potent and balanced dual 5-HT–norepinephrine reuptake inhibitor (Thor and Katofiasc, 1995, Mariappan et al., 2007), with effects on the urethral resistance during electrical stimulation clearly larger than those of duloxetine in rats. One of the possible explanations for the larger effects of TAK-233 may be that duloxetine stimulates both positively effective receptor subtypes, such as 5-HT$_{2C}$ receptor, 5-HT$_{7}$ receptor, and $x_1$-adrenoceptor (Miyazato et al., 2009, Suzuki et al., 2018, Karioi et al., 2007), and negatively effective receptor subtypes, such as 5-HT$_{1A}$ receptor and $x_2$-adrenoceptor (Yoshimura and Miyazato, 2012, Miyazato et al., 2008), that, when taken together, increase urethral function to an overall extent in rats. TAK-233, on the other hand, may only stimulate the positively effective receptor subtype 5-HT$_{2C}$ receptor with no stimulation of  

### Table 2

<table>
<thead>
<tr>
<th>Nerve transected</th>
<th>TAK-233</th>
<th>LPP</th>
<th>Pre</th>
<th>Post</th>
<th>Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/kg, intraduodenal</td>
<td>cmH$_2$O</td>
<td>cmH$_2$O</td>
<td>cmH$_2$O</td>
<td></td>
</tr>
<tr>
<td><strong>Sham</strong></td>
<td>0</td>
<td>54.1 ± 13.3</td>
<td>52.7 ± 13.5</td>
<td>1.4 ± 1.9</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>51.3 ± 3.4</td>
<td>76.9 ± 5.2</td>
<td>25.6 ± 3.5**</td>
<td></td>
</tr>
<tr>
<td><strong>Pelvic</strong></td>
<td>0</td>
<td>33.1 ± 6.1</td>
<td>34.6 ± 6.9</td>
<td>1.5 ± 1.5</td>
<td>#</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>30.0 ± 2.5</td>
<td>29.8 ± 3.6</td>
<td>0.3 ± 1.5</td>
<td>#</td>
</tr>
<tr>
<td><strong>Hypogastric</strong></td>
<td>0</td>
<td>40.8 ± 7.0</td>
<td>41.5 ± 5.9</td>
<td>0.7 ± 1.2</td>
<td>#</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>42.9 ± 6.3</td>
<td>69.0 ± 7.9</td>
<td>26.1 ± 4.2**</td>
<td>N.S.</td>
</tr>
<tr>
<td><strong>Pudendal</strong></td>
<td>0</td>
<td>33.8 ± 4.0</td>
<td>32.8 ± 3.5</td>
<td>1.0 ± 1.8</td>
<td>#</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>30.6 ± 3.1</td>
<td>38.9 ± 3.6</td>
<td>8.3 ± 2.1*</td>
<td>#</td>
</tr>
<tr>
<td><strong>Ilio/Pubo</strong></td>
<td>0</td>
<td>35.8 ± 4.9</td>
<td>32.7 ± 5.1</td>
<td>3.1 ± 1.2</td>
<td>#</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>41.9 ± 5.9</td>
<td>66.5 ± 11.7</td>
<td>24.6 ± 6.1**</td>
<td>N.S.</td>
</tr>
<tr>
<td><strong>Pudendal +</strong></td>
<td>0</td>
<td>27.0 ± 4.4</td>
<td>28.3 ± 4.5</td>
<td>1.3 ± 0.7</td>
<td>#</td>
</tr>
<tr>
<td><strong>Ilio/Pubo</strong></td>
<td>3</td>
<td>36.8 ± 6.9</td>
<td>43.0 ± 7.0</td>
<td>6.1 ± 2.5</td>
<td>#</td>
</tr>
</tbody>
</table>

In all rats, the spinal cord was transected at T8–T9 level. The pelvic nerves, the hypogastric nerves, the pudendal nerves, the nerves to iliococcygeus/pubococcygeus muscles (Ilio/Pubo), or both the pudendal nerves and Ilio/Pubo were bilaterally transected. Data are expressed as means ± S.E.M. in 5 rats.

N.S., not significant.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the vehicle-administered group in rats with the same nerves transected by Student’s $t$ test. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$: LPP increase after TAK-233 administration was compared with that in the sham-operated group by Dunnett’s test.
muscles to the urethral resistance. The abdominal muscles, contribution of the smooth and striated muscles during sneezing and electrical stimulation of the abdominal muscles in rats (Yoshimura and Miyazato, 2012, Kamo et al., 2003, 2004, 2009, Kamo and Hashimoto, 2007). In the current rat study, with electrical stimulation of the abdominal muscles, contribution of the smooth and striated muscles to the urethral resistance—increasing effects of TAK-233 were investigated by bilateral transection of the hypogastric nerves innervating the smooth urethral sphincter muscle (de Groat et al., 1993), the pudendal nerves innervating the external urethral sphincter and the coccygeus muscle (Manzo et al., 2000), and the nerves to iliococcygeus/pubococcygeus muscles (Pacheco et al. 1989, Kamo et al., 2003, 2004), respectively. The increasing effects were greatly suppressed by bilateral transection of both the pudendal nerves and the nerves to iliococcygeus/pubococcygeus muscles. In contrast, bilateral transection of the hypogastric nerves did not change the effect, suggesting a greater contribution of the striated muscles to the effects of TAK-233. In addition, since urethra-closing responses during sneezing are themselves totally abolished by bilateral transection of the nerves innervating the striated muscles (Kamo et al., 2003), it seems reasonable to consider that the urethral closure—increasing effects of TAK-233 during sneezing are also mediated by elevating striated muscle function. As striated muscle-mediated mechanisms greatly contribute to momentary reflex urethral closures (Kamo et al., 2009), the effects of TAK-233 may be more amenable to detect during momentary urethral closure than during continuous measurement.

It is considered highly likely that the site of action of TAK-233 for urethral closure—enhancing effects is located at Onuf’s nucleus in the sacral spinal cord similar to other 5-HT_{2C} receptor agonists (Yoshimura and Miyazato, 2012). Spinal 5-HT_{2C} receptor stimulation by intrathecal drug injection enhances urethra-closing responses in rats (Miyazato et al., 2009), and the current nerve transection study revealed that striated muscle, not smooth muscle, components greatly contributed to the effects of TAK-233, and the motor neurons innervating the striated external urethral sphincter via the pudendal nerves are located at Onuf’s nucleus (Rajaofetra et al., 1992, Thor, 2003, 2004). Furthermore, the reflex pathway from Onuf’s nucleus to the urethra is common between the two urethra-closing reflexes (Kamo et al., 2009, Yoshimura and Miyazato, 2012), and TAK-233 enhanced the urethra-closing function mediated by both types of reflexes. Therefore, it seems natural to conclude that TAK-233 acts on 5-HT_{2C} receptors on motor neurons in Onuf’s nucleus to enhance both types of reflexes, resulting in the elevation of urethral resistance under momentary urethral closure.

The effects of 5-HT_{2C} receptor stimulation on urethra-closing functions in healthy female humans were studied with TAK-233, with duloxetine as a reference drug known to reduce the frequency of stress urinary incontinence episodes in patients (Mariappan et al., 2007). Based on the putative

Fig. 5. Typical recordings of the middle urethral responses measured by microtip transducer catheter during increments of P_{ves} in rats with the spinal cord transection at the T8–T9 level. Responses are shown before (Pre) and 30 minutes after intraduodenal administration of TAK-233 at 3 mg/kg (Post).

Fig. 6. Time profiles of change from baseline in maximal urethral pressure at rest in the entire functional urethral length in healthy female subjects. Data are expressed as means ± S.D. of change from the baseline values.

Fig. 7. Time profiles of change from baseline in MT for urethral sphincter contraction in response to transcranial magnetic stimulation in healthy female subjects. Data are expressed as means ± S.D. of percent change from the baseline MT values. Means ± S.D. of baseline MT values were 65.0 ± 9.97, 66.3 ± 12.27, 70.0 ± 10.22, and 67.8 ± 11.46 (placebo, TAK-233 20 mg, TAK-233 90 mg, duloxetine 40 mg, respectively). + Changes from baseline were significantly lower than that in the placebo group by ANOVA.
mechanism of TAK-233, effects on the sensitivity of evoked urethral responses were primarily investigated by measuring stimulus threshold for inducing TMS-evoked momentary urethral sphincter contraction. Two published studies demonstrate that duloxetine increases the sensitivity of the evoked urethral responses (Boy et al., 2006; Yono et al., 2015), suggesting this as one potential mechanism for a drug for the treatment of stress urinary incontinence. Our pharmacodynamic study in humans obtained the same finding, with TAK-233 significantly lowering the stimulus threshold even at the lower dose (20 mg) and those effects generally following the time course of its pharmacokinetic profile. These findings indicate that TAK-233 increased the sensitivity of the evoked momentary urethra-closing mechanism and the 5-HT$_{2C}$ receptor-mediated enhancing mechanism functions in humans.

Compared with the effects of TAK-233 on the stimulus threshold, its effect on urethral pressure at rest required higher plasma drug levels. Although TAK-233 plasma levels of 125 ng/ml after administration at 90 mg were required for increasing the maximum urethral pressure at rest, a plasma level around 30 ng/ml after administration at 20 mg was enough to increase the sensitivity of the evoked momentary contractions. It seems reasonable to consider that the more the urethra-closing reflex pathway is stimulated, such as during the evoked urethral contractions, the more the effects of 5-HT$_{2C}$ receptor stimulation become obvious, and that the reflex does not function to any great extent during urethral pressure profile measurement at rest since the bladder was emptied before measurement of the reflex. Recently, another 5-HT$_{2C}$ receptor agonist, ASP2205, demonstrated no positive effects on the opening urethral pressure, both at rest and with squeezing, in healthy female subjects (Klarskov et al., 2019). Healthy subjects may show maximal striated muscle–mediated closure during squeezing even without drug administration, and duloxetine might increase smooth muscle–mediated adrenergic urethral closure mechanism.

As shown in the current human pharmacodynamic study, the time profile of change in evoked urethral closure–enhancing effects of each drug was generally consistent with the time course of the plasma drug levels, indicating that exceeding a plasma level threshold is necessary for the pharmacodynamic effects of respective drugs. Meanwhile, the required duration of an effective treatment may vary depending on each patient with stress urinary incontinence and their situation. For example, one patient may only need a short effective duration while engaging in daily activities for short periods of time, whereas another patient may need a longer effective duration to cover a full working day. The plasma levels of TAK-233 rapidly elevated after oral administration and then sharply declined after reaching its peak level. This pharmacokinetic profile, such as that demonstrated for TAK-233, in combination with slow-release formulation technology, etc., may make it possible to provide various medicines with different effective durations on a flexible basis, since in almost all cases, rapid achievement of the plasma level threshold is required.

The current studies with TAK-233 in female rats and humans demonstrate that 5-HT$_{2C}$ receptor stimulation enhances striated urethra-closing functions during both evoked and momentary events, and consequently, 5-HT$_{2C}$ receptor agonists may provide effective treatment options for patients with stress urinary incontinence. However, the effects of 5-HT$_{2C}$ receptor stimulation on the frequency of stress urinary incontinence episodes remain unclear, requiring further clinical studies.

### Authorship Contributions

**Participated in research and study design:** Kamo, Nagata, O’Connell, Kato, Imamishi, Yoshikawa, Nishiyama.

**Conducted experiments and the study:** Nagata, Kato, Imamishi, Kuno, Okanishi, Nishiyama.

**Performed data analysis:** Kamo, Nagata, O’Connell, Kato, Imamishi, Kuno, Okanishi, Yoshikawa, Nishiyama.

**Wrote or contributed to the writing of the manuscript:** Kamo, Imamishi, Nishiyama.

### Acknowledgments

We would like to thank project chemists, especially Shigekazu Sasaki, Tomokazu Kasumoto, Izumi Nomura, and Hirosho Mera as TAK-233 inventors, for identifying TAK-233 after their great efforts in medicinal chemistry studies. We would like to thank the investigator and other staff members for dedicated operation for the clinical study.

**Note Added in Proof:** During the proof stage, Table 3 layout was changed to help with readability from what was published in the Fast Forward version that appeared online April 9, 2021. Table 3 has now been updated.

### References


### Table 3

Plasma concentration alteration of TAK-233 and duloxetine in healthy female subjects

<table>
<thead>
<tr>
<th>Time</th>
<th>TAK-233 20 mg</th>
<th>TAK-233 90 mg</th>
<th>Duloxetine 40 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>0.5 hr</td>
<td>29.2 ± 10.2</td>
<td>127.3 ± 79.2</td>
<td>-</td>
</tr>
<tr>
<td>1 hr</td>
<td>25.2 ± 10.4</td>
<td>126.0 ± 59.2</td>
<td>-</td>
</tr>
<tr>
<td>2 hr</td>
<td>20.4 ± 8.7</td>
<td>152.8 ± 68.1</td>
<td>-</td>
</tr>
<tr>
<td>3 hr</td>
<td>14.3 ± 6.7</td>
<td>122.4 ± 52.4</td>
<td>6.0 ± 7.3</td>
</tr>
<tr>
<td>6 hr</td>
<td>3.5 ± 2.0</td>
<td>52.8 ± 31.4</td>
<td>32.4 ± 15.7</td>
</tr>
<tr>
<td>8 hr</td>
<td>1.4 ± 0.8</td>
<td>24.7 ± 16.5</td>
<td>29.2 ± 13.0</td>
</tr>
<tr>
<td>12 hr</td>
<td>0.1 ± 0.3</td>
<td>5.6 ± 4.5</td>
<td>21.1 ± 9.9</td>
</tr>
<tr>
<td>24 hr</td>
<td>-</td>
<td>-</td>
<td>9.4 ± 5.8</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SD in 23 subjects. - indicates not tested.


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