1521-0103/373/2/239–247$35.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

https://doi.org/10.1124/jpet.119.263616
J Pharmacol Exp Ther 373:239–247, May 2020

The Journal of PHARMACOLOGY And Experimental Therapeutics

KPR-5714, a Novel Transient Receptor Potential Melastatin 8 Antagonist, Improves Overactive Bladder via Inhibition of Bladder Afferent Hyperactivity in Rats

Osamu Nakanishi,[1] Yoshikazu Fujimori,[1] Naoki Aizawa,[2] Takemitsu Hayashi, Akane Matsuzawa, Jun-ichi Kobayashi, Hideaki Hirasawa, Yosuke Mutai, Fumiya Tanada, and Yasuhiko Igawa[3]

Discovery Research R&D, Kissei Pharmaceutical Co., Ltd., Azumino, Japan (O.N., Y.F., T.H., A.M., JI.K., H.H., Y.M., F.T.) and Department of Continence Medicine, the University of Tokyo Graduate School of Medicine, Tokyo, Japan (N.A., Y.I.)

Received November 6, 2019; accepted February 24, 2020

ABSTRACT

Transient receptor potential (TRP) melastatin 8 (TRPM8) is a temperature-sensing ion channel mainly expressed in primary sensory neurons (Aδ-fibers and C-fibers in the dorsal root ganglion). In this report, we characterized KPR-5714 (*N*-[(*R*)-3,3-difluoro-4-hydroxy-1-(2*H*-1,2,3-triazol-2-yl)butan-2-yl]-3-fluoro-2-[5-(4-fluorophenyl)-1*H*-pyrazol-3-yl]benzamide), a novel and selective TRPM8 antagonist, to assess its therapeutic potential against frequent urination in rat models with overactive bladder (OAB). In calcium influx assays with HEK293T cells transiently expressing various TRP channels, KPR-5714 showed a potent TRPM8 antagonistic effect and high selectivity against other TRP channels. Intravenously administered KPR-5714 inhibited the hyperactivity of mechanosensitive C-fibers of bladder afferents and dose-dependently increased the intercontraction interval shortened by intravesical instillation of acetic acid in anesthetized rats. Furthermore, we examined the effects of KPR-5714 on voiding behavior in conscious rats with cerebral infarction and in those exposed to cold in metabolic cage experiments. Cerebral infarction and cold exposure induced a significant decrease in the mean voided volume and increase in voiding frequency in rats. Orally administered KPR-5714 dose-dependently increased the mean voided volume and decreased voiding frequency without affecting total voided volume in these models. This study demonstrates that KPR-5714 improves OAB in three different models by inhibiting exaggerated activity of mechanosensitive bladder C-fibers and suggests that KPR-5714 may provide a new and useful approach to the treatment of OAB.

SIGNIFICANCE STATEMENT

TRPM8 is involved in bladder sensory transduction and plays a role in the abnormal activation in hypersensitive bladder disorders. KPR-5714, as a novel and selective TRPM8 antagonist, may provide a useful treatment for the disorders related to the hyperactivity of bladder afferent nerves, particularly in overactive bladder.

Introduction

Transient receptor potential (TRP) channels are nonselective cation channels that are activated by environmental and endogenous stimuli, such as temperature, osmotic pressure, chemical substances, and mechanical stimulus (Clapham, 2003). TRP melastatin 8 (TRPM8) is a temperature-sensing TRP channel that can be activated by innocuous cold stimuli and chemical substances such as menthol and icilin (McKemy et al., 2002; Peier et al., 2002). TRPM8 is highly expressed in a subset of sensory Aδ-fiber and C-fiber dorsal root ganglion neurons and in trigeminal sensory neurons (Kobayashi et al., 2005), and activation of these sensory neurons via TRPM8 induces the sensation of cold (Bautista et al., 2007).

The lower urinary tract (LUT) consists of the bladder and the urethra, two functional units for storage and elimination of urine. The LUT is densely innervated by afferent and efferent fibers and constantly sends mechanosensory information to the central nervous system via the afferent pathway (Fowler et al., 2008; Yoshimura et al., 2008; Kanai and Andersson, 2010). These signals generate filling sensation and trigger voiding responses. In pathologic conditions, such as detrusor overactivity and overactive bladder (OAB), the chemical and electrical properties of bladder afferent pathways are altered, resulting in urinary urgency and increased voiding frequency (Fowler et al., 2008; Yoshimura et al., 2008; Kanai and Andersson, 2010).

TRPM8 is involved in nociception and mechanosensory transduction in the LUT, and its potential as a urological

This research was funded by Kissei Pharmaceutical Co., Ltd. O.N., Y.F., T.H., A.M., JI.K., H.H., Y.M., and F.T. are employees of Kissei Pharmaceutical Co., Ltd.
[1]O.N. and Y.F. contributed equally to this work.
[2]Current affiliation: Department of Pharmacology and Toxicology, Dokkyo Medical University School of Medicine, Mibu, Tochigi, Japan.
[3]Current affiliation: Department of Urology, Nagano Prefectural Shinshu Medical Center, Suzaka City, Nagano, Japan.
https://doi.org/10.1124/jpet.119.263616.

ABBREVIATIONS: CV, conduction velocity; KPR-5714, *N*-[(*R*)-3,3-difluoro-4-hydroxy-1-(2*H*-1,2,3-triazol-2-yl)butan-2-yl]-3-fluoro-2-[5-(4-fluorophenyl)-1*H*-pyrazol-3-yl]benzamide; LUT, lower urinary tract; OAB, overactive bladder; SAA, single-unit afferent activity; TRP, transient receptor potential; TRPM8, TRP melastatin 8.

treatment target has been assessed (Skryma et al., 2011; Andersson, 2019). TRPM8 immunostaining is observed in human suburothelial nerve fibers, with a marked increase in TRPM8-immunoreactive unmyelinated C-fibers in bladder specimens from patients with idiopathic detrusor overactivity and painful bladder syndrome (Mukerji et al., 2006). Furthermore, the increased density of TRPM8-immunoreactive unmyelinated nerve fibers is correlated with voiding frequency and pain scores (Mukerji et al., 2006), suggesting that the inhibition of the TRPM8 could be an effective treatment of bladder overactivity and bladder pain.

To date, a number of TRPM8 antagonists have been reported (Pérez de Vega et al., 2016; González-Muñiz et al., 2019). Intravenous administration of *N*-(3-aminopropyl)-2-{[(3-methylphenyl)methyl]oxy}-*N*-(2-thienylmethyl)benzamide hydrochloride (AMTB) decreases the frequency of volume-induced bladder contractions and nociceptive reflex responses to noxious urinary bladder distension in rats (Lashinger et al., 2008). However, AMTB induces low blood pressure at a high effective dose in rats (Lashinger et al., 2008). We have previously reported that KPR-2579 has a sufficient safety margin for cardiovascular effects, reduces frequency of rhythmic bladder contractions without affecting the amplitude of contractions (Kobayashi et al., 2017), and inhibits acetic acid-induced bladder afferent hyperactivity in rats (Aizawa et al., 2018). Another recent study suggested that RQ-00434739 (structure not disclosed) inhibits L-menthol and prostaglandin E2-induced hyperactivity of the primary bladder afferent nerves in rats (Aizawa et al., 2019). Therefore, TRPM8 may be involved in the bladder sensory transduction and play an important role in the abnormal activation in bladder afferent pathways mediated via mechanosensitive C-fibers.

In this report, we describe a potent and selective TRPM8 antagonist, KPR-5714, whose structural scaffold is different from that of KPR-2579. We confirmed the inhibitory effects of KPR-5714 on bladder afferent hyperactivity provoked by intravesical acetic acid instillation. Subsequently, to assess the therapeutic potential of KPR-5714 for OAB-associated facilitation of bladder afferent activity, we examined the effect of KPR-5714 on the voiding behavior of rats with cerebral infarction and those exposed to cold.

Materials and Methods

Chemicals. KPR-5714 (*N*-[(*R*)-3,3-difluoro-4-hydroxy-1-(2*H*-1,2,3-triazol-2-yl)butan-2-yl]-3-fluoro-2-[5-(4-fluorophenyl)-1*H*-pyrazol-3-yl] benzamide) and mirabegron were synthesized by Kissei Pharmaceutical Co., Ltd. (Nagano, Japan). Tolterodine tartrate was purchased from ChemPacific Corp. (Baltimore, MD). Menthol, allyl isothiocyanate, capsaicin, RN-1747, *N*,*N*-dimethylacetamide, and acetic acid were obtained from Wako Pure Chemical Industries (Tokyo, Japan).

Molecular Properties. Molecular weight, calculated logarithm of octanol/water partition coefficient, topological polar surface area, and lipophilic efficiency of KPR-5714 were calculated using Marvin calculator plugins (ChemAxon, Budapest, Hungary).

Calcium Influx Assay. The cDNA of human TRPM8 (NM_024080.4), TRPA1 (NM_007332.2), TRPV1 (NM_080704.3), and TRPV4 (NM_021625.4) and rat TRPM8 (NM_134371.2) were inserted into a pcDNA3.3 (TRPM8), pcDNA3.1 (TRPA1, TRPV4), or pCI-neo (TRPV1) expression plasmid. The calcium influx assay was performed as previously described (Kobayashi et al., 2017). In this experiment, EC_{80} concentration of menthol, allyl isothiocyanate, capsaicin, and RN-1747 were used as agonists for TRPM8, TRPA1, TRPV1, and TRPV4, respectively. An agonist-induced increase in intracellular calcium level was measured using an FDSS plate reader (Hamamatsu Photonics, Hamamatsu, Japan). The IC_{50} values were calculated from the concentration-response curve with nonlinear regression analysis using GraphPad Prism 4.0 (GraphPad Software, Inc., La Jolla, CA).

Animals. Female Sprague-Dawley rats (Charles River, Yokohama, Japan; and Japan SLC, Inc., Shizuoka, Japan) were used. Rats were maintained under standard controlled conditions with a 12-hour lighting cycle and access to chow and water ad libitum. Experimental protocols were approved by the Laboratory Animal Committee of Kissei Pharmaceutical Co., Ltd. and the Institutional Animal Care and Use Committee of the University of Tokyo.

Drug Preparation. For intravenous administration, 1 ml/kg of KPR-5714 was dissolved in a 20% *N*,*N*-dimethylacetamide and 80% saline solution. For oral administration, 5 ml/kg of KPR-5714, tolterodine tartrate, or mirabegron were dissolved in a 5% *N*,*N*-dimethylacetamide and 0.5% methyl cellulose 400 solution (95%; Wako Pure Chemical Industries).

Afferent Activity Measurements. Rats were anesthetized with urethane (1.2 g/kg intraperitoneally). The measurement of single-unit afferent activities (SAAs) was performed as previously described (Aizawa et al., 2018). In brief, after a pair of silver stimulation electrodes was placed around the left pelvic nerve, both L6 dorsal roots were cut near their entrance into the spinal cord. Unitary action potentials of mechanosensitive bladder afferent nerve fibers isolated from the left L6 dorsal root were identified as SAAs by electrical stimulation of the pelvic nerve and by bladder distension with saline via an inserted bladder catheter (PE-50; Clay Adams, Parsippany, NJ). The SAAs were grouped on the basis of their conduction velocity (CV); that is, those with a CV of <2.5 m/s were considered to correspond to C-fibers, whereas those with a CV of ≥ 2.5 m/s were considered to correspond to Aδ-fibers. SAAs and intravesical pressure were recorded during constant saline instillation (6 ml/h) into the bladder. The filling continued until an intravesical pressure of 30 cmH_2O was achieved. SAAs were expressed as the firing rates (Hertz) and evaluated in relation to intravesical pressure at each 5-cm H_2O interval. Average SAAs were totaled as a function of intravesical pressure. At the beginning of the experiments, recording was repeated two or three times consecutively at 5-minute intervals to evaluate reproducibility. Subsequently, vehicle or KPR-5714 was intravenously administered. Then, 5 minutes after drug administration, the recording was repeated three times during 0.1% acetic acid instillation.

Cystometry Measurements. Rats were anesthetized with urethane (1.2 g/kg i.p.). A lower abdominal midline incision was made to expose the bladder, and a PE-50 catheter was inserted into the bladder dome. Another PE-50 catheter was placed in the left jugular vein for drug administration. To record the intravesical pressure, the intravesical catheter was connected via a three-way stopcock to a pressure transducer (DX-100; Nihon Kohden, Tokyo, Japan), and a syringe pump (KDS 100; Muromachi Kikai Co. Ltd., Tokyo, Japan) was used for instillation of saline or 0.25% acetic acid (3.6 ml/h). Intravesical pressure was recorded on a rectigraph (Recti-Horiz-8K; San-ei Instruments Inc., Tokyo, Japan). After confirming a stable intercontraction interval by intravesical instillation of saline, 0.25% acetic acid was instilled. The intercontraction interval was decreased with acetic acid instillation by more than 40% compared with saline instillation, and vehicle or KPR-5714 was intravenously administered. Intercontraction intervals and micturition pressures were obtained from three micturition cycles before dosing (baseline) and after dosing, and the ratio to baseline was calculated.

Voiding Behavior Measurements Using Metabolic Cages. To evaluate voiding behavior, rats were individually placed in a metabolic cage (Tecniplast Japan Co. Ltd., Tokyo, Japan) mounted on an electrical balance (A&D Company, Ltd., Tokyo, Japan). The balance was connected to a computer, and data on voided urine was continuously collected. A change in the urine weight (>0.1 g) was

regarded as a voiding episode, and the specific gravity of urine was defined as 1 g/ml. Vehicle, KPR-5714, tolterodine tartrate, or mirabegron was orally administered, and rats were acclimated to the metabolic cage. One hour after drug administration, rats were orally loaded with water at a volume of 4 ml/body, and the cumulative voided volume was recorded on a computer at 2-minute intervals for 4 hours. The mean voided volume, voiding frequency, and total voided volume were calculated.

Induction of Cerebral Infarction. Under isoflurane anesthesia, cerebral infarction was induced by middle cerebral artery occlusion according to the method described by Longa et al. (1989) and Kaidoh et al. (2002). The left carotid bifurcation was exposed through a midline incision, and both the left common carotid and the left external carotid arteries were ligated near the bifurcation. The left internal carotid was carefully exposed, and the left pterygopalatine branch was ligated close to its origin. A 4-0 monofilament nylon thread (Keisei Medical Industrial Co., Ltd., Tokyo, Japan) with its tip coated with silicon (Shin-Etsu Chemical Co., Ltd., Tokyo, Japan) was inserted into the middle cerebral artery approximately 17 mm from the carotid bifurcation. One day after the middle cerebral artery occlusion, voiding behavior of cerebral infarcted rats was evaluated as described above. At the end of the experiments, rats were sacrificed to confirm the formation of a cerebral ischemic lesion. The brain was removed and cut into 2-mm coronal sections that were further immersed in 2% 2,3,5-triphenyl tetrazolium chloride (Wako Pure Chemical Industries). Rats with no cerebral infarction were excluded from this study.

Frequent Urination Induced by Cold Exposure. Vehicle, KPR-5714, tolterodine tartrate, or mirabegron was orally administered, and rats were acclimated to the metabolic cage at room temperature (23–26°C). One hour after drug administration, rats were orally loaded with water at a volume of 4 ml/body and transferred to the metabolic cage placed in the low temperature incubator (Fukushima Industries Corp., Osaka, Japan) regulated at 4°C. Then the cumulative voided volume was recorded on a computer at 2-minute intervals for 4 hours, and the voiding behavior of rats exposed to cold was evaluated as described above.

Statistical Analysis. Data were presented as means ± S.E.M. for each group. Statistical analyses were performed using SAS Systems Version 9.3 (SAS Institute, Inc., Cary, NC) and GraphPad Prism Version 6 (GraphPad Software). Equality of variances were first analyzed with F-test or Bartlett's test, and subsequently, statistical significance was determined with Aspin-Welch t test, Student's t test, Steel multiple comparison test, or Dunnett's multiple comparison test as appropriate. Results of afferent activity measurements were analyzed using unpaired t tests for comparisons between groups at each time or repeated-measures analysis of variance following Dunnett's test for comparison before and after drug administration in each group.

Results

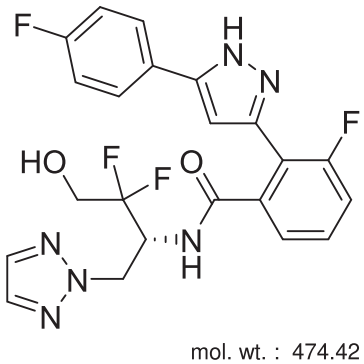
Inhibitory Effects on Human and Rat TRP Channels. The structure of KPR-5714 is shown in Fig. 1. The inhibitory effects of KPR-5714 on calcium influx into HEK293T cells expressing human or rat TRP channels, given in terms of IC_{50}, are shown in Table 1. KPR-5714 showed potent antagonistic effects against human and rat TRPM8, with IC_{50} values of 25.3 and 22.4 nM, respectively. Selectivity of KPR-5714 for TRPM8 was approximately 400-fold against human TRPA1, TRPV1, and TRPV4, and KPR-5714 did not show inhibitory effects for human acid-sensing ion channel (ASIC) 1a, ASIC3, voltage-gated sodium channel (Nav) 1.3, Nav1.5, Nav1.6, Nav1.7, and Nav1.8, with IC_{50} values of >10 μM (data not shown). These results suggest that KPR-5714 is a potent and selective TRPM8 antagonist.

Inhibitory Effects on Bladder Afferent Hyperactivity. A total of 48 single afferent fibers (n = 24 in each fiber, CVs: Aδ-fibers: 5.06 ± 0.55 m/s, C-fibers: 2.00 ± 0.08 m/s) were isolated from 42 rats. In the presence of vehicle, acetic acid instillation significantly increased the SAAs of C-fibers, whereas SAAs of Aδ-fibers were not significantly changed. Pretreatment with KPR-5714 at 0.1 mg/kg significantly inhibited the acetic acid-induced activation of C-fiber SAAs (Figs. 2 and 3B). In contrast, the same KPR-5714 dose (0.1 mg/kg) decreased SAAs of Aδ-fibers compared with baseline in a time-dependent manner, which was significant at a later phase (after 3). These changes in the group treated with KPR-5714 0.1 mg/kg were also significant for comparison with the vehicle-treated group (Fig. 3A).

Effects on Cystometric Parameters in Rats with Acetic Acid-Induced Bladder Overactivity. Acetic acid (0.25%) instillation into the bladder showed significant decrease in intercontraction interval (Fig. 4A). Vehicle had no effect on intercontraction interval (Fig. 4B), and KPR-5714 at 1 mg/kg increased the intercontraction interval (Fig. 4C) during acetic acid instillation. KPR-5714 dose-dependently increased the intercontraction interval (Fig. 5A) without reducing micturition pressure (Fig. 5B) in rats with acetic acid-induced bladder overactivity.

Effects on Voiding Behavior in Rats with Cerebral Infarction. Representative traces of voided volumes in normal and cerebral infarcted rats are shown in Fig. 6. Compared with normal rats, cerebral infarcted rats showed a significant decrease in mean voided volume and a significant increase in voiding frequency (Table 2). In the cerebral infarcted rats, oral administration of KPR-5714 dose-dependently increased the mean voided volume and decreased voiding frequency without affecting total voided volume (Table 2). Tolterodine tartrate did not show a significant change in voiding behavior. Mirabegron increased the mean voided volume and showed a tendency to decrease voiding frequency (P = 0.052) without affecting total voided volume.

Effects on Voiding Behavior in Rats Exposed to Cold. Representative traces of voided volumes in rats under the condition of room temperature and 4°C are shown in Fig. 7. Rats exposed to cold showed a significant decrease in mean voided volume and a significant increase in voiding frequency and total voided volume compared with rats under room temperature (Table 3). Oral administration of KPR-5714 and



mol. wt. : 474.42
cLogP : 2.86
tPSA : 108.72
LipE : 4.74

Fig. 1. Chemical structure and molecular properties of KPR-5714.

TABLE 1

IC_{50} values of KPR-5714 for human and rat TRP channels

Human or rat TRP channel expression plasmid was transfected into HEK293T cells. Calcium influx assay was performed using transiently transfected cells, and the IC_{50} values for each TRP channel were calculated.

	IC_{50} value (nM)				
	Human TRPM8	Rat TRPM8	Human TRPA1	Human TRPV1	Human TRPV4
KPR-5714	25.3 ± 2.5[a]	22.4 ± 3.1[a]	>10,000[b]	>10,000[b]	>10,000[c]

[a]Data are presented as means ± S.E.M. from four experiments.
[b]Data are presented from three experiments.
[c]Data are obtained from one experiment.

tolterodine tartrate significantly increased the mean voided volume and decreased voiding frequency without affecting the total voided volume (Table 3). Mirabegron resulted in no significant change in voiding behavior of rats exposed to cold.

Discussion

Several members of the TRP channel are involved in nociception and mechanosensory transduction in the LUT and have been evaluated as urological treatment targets in animal experiments. Among them, we had focused on TRPM8 and reported another TRPM8 antagonist, KPR-2579 (human TRPM8: IC_{50} = 80 nM, selectivity over other TRP channels: >375-fold), as a tool for analyzing the role of TRPM8 in bladder function (Kobayashi et al., 2017). To overcome problems with KPR-2579, we have optimized the lead compound of which scaffold is different from KPR-2579 and finally discovered KPR-5714 as a potent and selective TRPM8 antagonist (human TRPM8: IC_{50} = 25.3 nM, selectivity over other TRP channels: approximately 400-fold, Table 1). In our previous study, KPR-2579 dose-dependently reduced the number of icilin-induced wet-dog shakes in rats (31%, 73%, and 100% inhibition for 1, 3, and 10 mg/kg group, respectively) (Kobayashi et al., 2017). In contrast, the inhibition rates of wet-dog shakes by KPR-5714 were 29% at 0.1 mg/kg and 97% at 0.3 mg/kg (data not shown). Based on IC_{50} values and inhibitory effect on icilin-induced wet-dog shakes, we confirmed that KPR-5714 is a potent and highly selective TRPM8 antagonist. For better understanding of inhibitory mechanism against TRPM8, further investigation of the electrophysiological profile of KPR-5714 will be required, and this is a limitation of our present study.

We previously reported that TRPM8 may have physiologic and pathophysiological roles in activating bladder afferent pathways mediated via mechanosensitive C-fibers (Aizawa et al., 2018). In the present study, we first used acetic acid, which stimulates sensory neurons that form the afferent arm of the micturition reflex and shortens the voiding intervals (Woods et al., 2001). In fact, we confirmed the inhibitory effects of KPR-5714 on bladder afferent hyperactivity of C-fibers induced by acetic acid instillation into the bladder (Figs. 2 and 3B). KPR-5714 at 1 mg/kg exhibited a 178% elongation of the intercontraction interval without reducing micturition pressure (Fig. 5). These results suggest that the effect of KPR-5714 on the intercontraction interval in rats with acetic acid-induced bladder overactivity could be mediated via inhibition of bladder C-fiber hyperactivity. Edmondson et al. (2016) reported that the novel β3-adrenoceptor

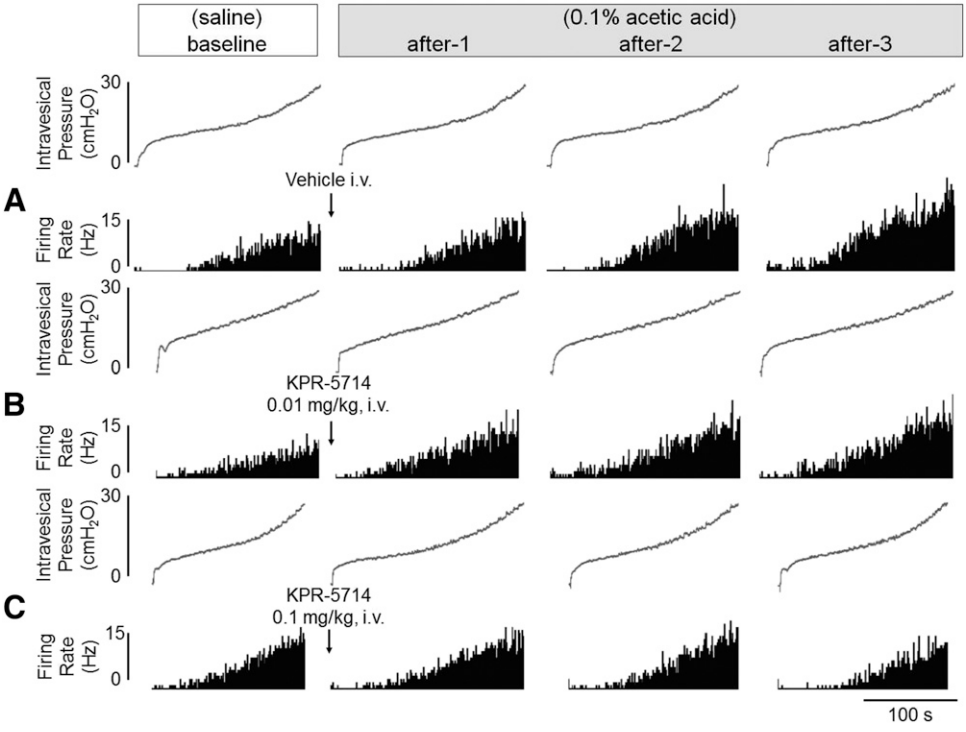


Fig. 2. Representative recordings of the intravesical pressure and firing rates of C-fiber SAAs at baseline with saline instillation and during 0.1% acetic acid instillation after vehicle (A) or KPR-5714 0.01 mg/kg (B) and 0.1 mg/kg (C) intravenous administration.

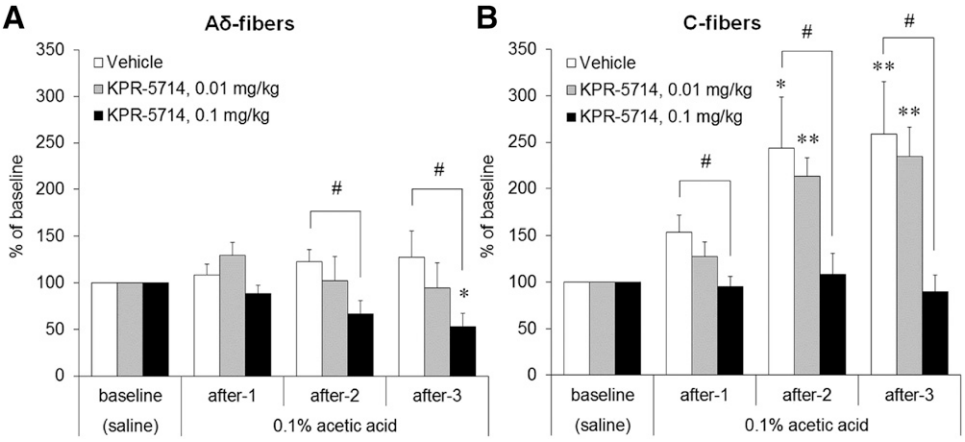


Fig. 3. Effects of KPR-5714 intravenous administration on SAAs of Aδ-fibers (A) and C-fibers (B) during 0.1% acetic acid instillation into the bladder. Data are presented as means ± S.E.M. (n = 8). $^{*}P < 0.05$; $^{**}P < 0.01$ from baseline in each group (repeated-measures analysis of variance followed by Dunnett's test); $^{\#}P < 0.05$ from vehicle-treated group at each time point (unpaired Student's t test).

agonist, vibegron, exhibited a dose-dependent decrease in micturition pressure and an increase in functional bladder capacity in the acetic acid instillation model. KPR-5714 produced a similar degree of elongation effect on the intercontraction interval as vibegron. The β3-adrenoceptor agonist shows relaxant activity in isolated rat bladder smooth muscle, whereas KPR-5714 at 1 μM did not show relaxant effects (data not shown). In a recent study, RQ-00434739, another selective TRPM8 antagonist, inhibited prostaglandin E2-induced bladder overactivity via inhibiting the hyperactivity of C-fiber SAAs (Aizawa et al., 2019). These previous results and the present study indicated that the TRPM8 antagonist may ameliorate abnormally exaggerated bladder afferent transduction. In the present study, KPR-5714 at 0.1 mg/kg also decreased SAAs of Aδ-fibers in a time-dependent manner (Fig. 3A). To our knowledge, this is the first report on the inhibitory effect of TRPM8 antagonist on Aδ-fiber SAAs in rats. As TRPM8 are highly expressed in a subset of not only C-fiber but also Aδ-fiber in the dorsal root ganglion neurons (Kobayashi et al., 2005), our results may give a new insight into the possible treatment mechanism by the TRPM8 antagonist, although this point may need to be assessed in further studies.

Frequent voiding and urinary incontinence are often observed in patients with cerebral infarction. It has been demonstrated that both anticholinergic drugs and β3-adrenoceptor agonists increase the mean voided volume in cerebral infarcted rats (Kaidoh et al., 2002; Suzuki et al., 2005; Hatanaka et al., 2013). In addition, a selective α1A-blocker improves bladder storage function in this model via suppression of

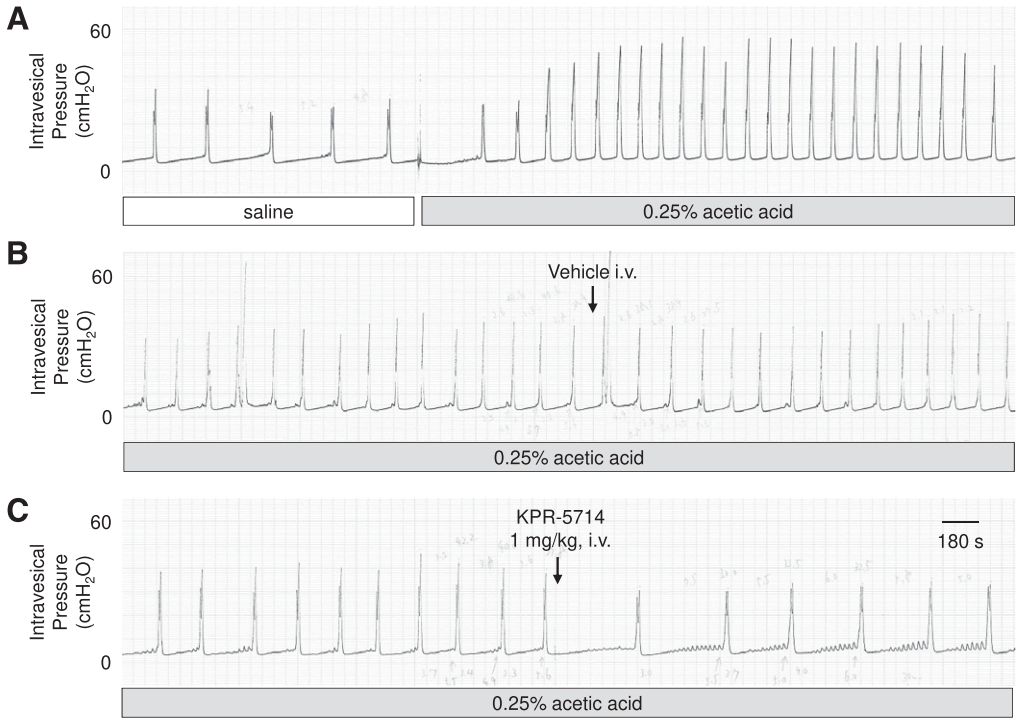


Fig. 4. Representative recordings of the cystometry measurements. After confirming stable intercontraction interval by intravesical instillation of saline, 0.25% acetic acid was instilled at the same instillation rate (A). Vehicle (B) or KPR-5714 at 1 mg/kg (C) was intravenously administered during 0.25% acetic acid instillation.

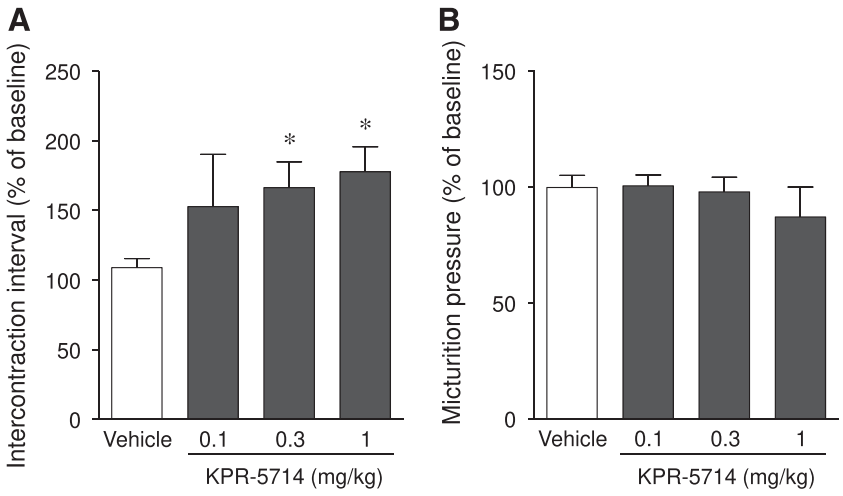


Fig. 5. Effect of KPR-5714 intravenous administration on intercontraction interval (A) and micturition pressure (B) in acetic acid-induced bladder overactivity. Percent of baseline was calculated as ratio between postdose values and predose values. Data are presented as means ± S.E.M. (n = 5). *$P < 0.05$ vs. vehicle group.

resiniferatoxin-sensitive C-fiber afferent activity (Yokoyama et al., 2010). We evaluated the effects of KPR-5714 on the voiding behavior in cerebral infarcted rats. Mirabegron (3 mg/kg) increased the mean voided volume in these rats, and our results are in line with a previous report (Hatanaka et al., 2013). KPR-5714 dose-dependently increased the mean voided volume and decreased voiding frequency without affecting total voided volume (Table 2). Yokokawa et al. (2017) revealed that bladder C-fibers are activated by cerebral infarction, and C-fiber afferent nerves play an important role on the secretion of nerve growth factor; thus, we supposed that the improvement of the voiding behavior by KPR-5714 may be also mediated via inhibition of bladder C-fiber hyperactivity similar to the case of acetic acid instillation.

TRPM8 is a sensor of environmental cold temperatures. It is known that low environmental temperature elicits more frequent urination in healthy individuals and influences bladder function in patients with LUT disorders (Watanabe et al., 2007), while cold weather aggravates symptoms in OAB patients (Ghei and Malone-Lee, 2005). In conscious rats, exposure to cold temperature decreased voiding interval and bladder capacity, and it has been suggested that these changes are mediated, at least in part, through resiniferatoxin-sensitive C-fiber afferent nervous pathways (Imamura et al., 2008).

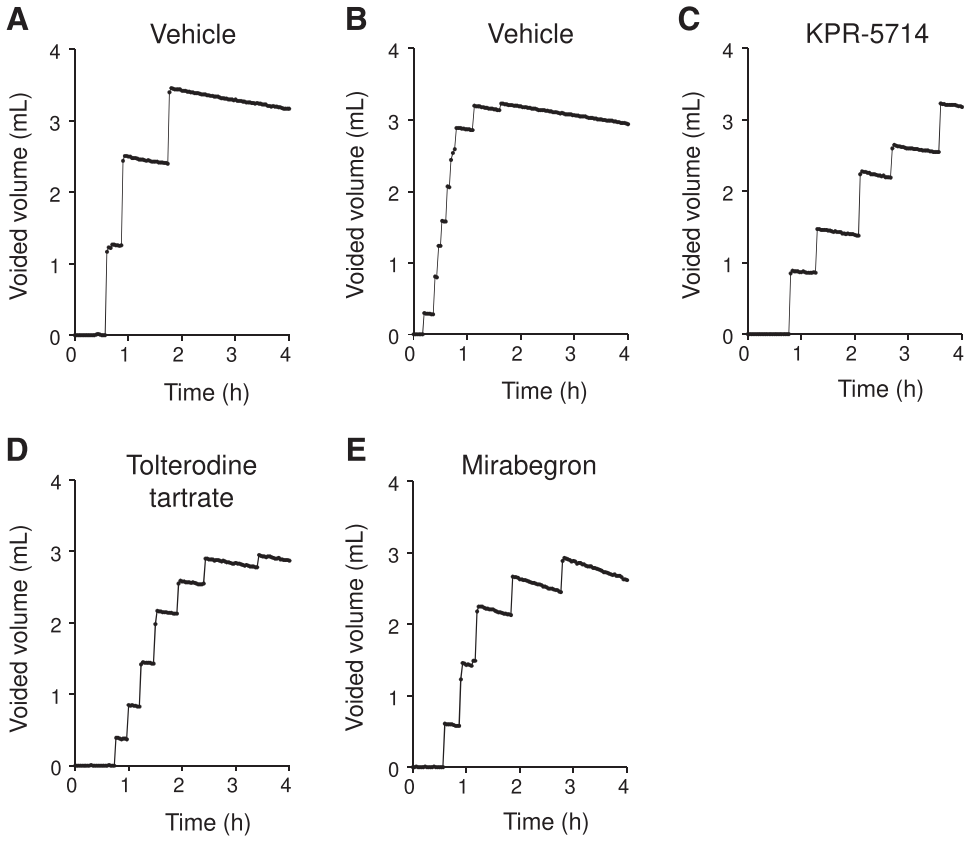


Fig. 6. Representative traces of frequency volume chart in normal rats given vehicle (A) and cerebral infarcted rats given vehicle (B), KPR-5714 1 mg/kg (C), tolterodine tartrate 10 mg/kg (D), and mirabegron 10 mg/kg (E). Vehicle, KPR-5714, tolterodine tartrate, or mirabegron was orally administered.

TABLE 2

Effects of KPR-5714 on voiding behavior in rats with cerebral infarction

Data are presented as means ± S.E.M. Vehicle, KPR-5714, tolterodine tartrate, or mirabegron was orally administered. Mean voided volume, voiding frequency, and total voided volume after drug administration was evaluated during the metabolic cage experiments. ##$P < 0.01$ vs. vehicle-treated normal rats, *$P < 0.05$; **$P < 0.01$ vs. vehicle-treated cerebral infarcted rats.

Animal	Compound	Dose (mg/kg)	n	Mean voided volume (ml)	Voiding frequency	Total voided volume (ml)
Normal	Vehicle		6	1.10 ± 0.12	3.3 ± 0.4	3.49 ± 0.30
Cerebral infarction	Vehicle		7	0.45 ± 0.04##	8.3 ± 0.6##	3.66 ± 0.33
	KPR-5714	0.3	6	0.62 ± 0.13	6.8 ± 0.6	3.89 ± 0.43
		1	5	0.70 ± 0.05*	5.4 ± 0.5**	3.67 ± 0.14
Cerebral infarction	Vehicle		6	0.41 ± 0.02##	8.5 ± 0.4##	3.45 ± 0.19
	Tolterodine tartrate	3	7	0.57 ± 0.15	7.9 ± 1.2	3.53 ± 0.34
		10	9	0.53 ± 0.07	7.2 ± 0.8	3.37 ± 0.13
	Mirabegron	3	7	0.56 ± 0.08*	6.7 ± 0.8	3.45 ± 0.24
		10	7	0.61 ± 0.05*	6.1 ± 0.7	3.60 ± 0.24

[a]$P < 0.01$ vs. vehicle-treated normal rats.
[b]$P < 0.05$.
[c]$P < 0.01$ vs. vehicle-treated cerebral infarcted rats.

In addition, application of cold stimuli or menthol to the skin, which induces the sensation of cold, increased bladder overactivity and voiding frequency in rats, and these responses were prevented by treatment with TRPM8 antagonists (Lei et al., 2013; Uvin et al., 2015; Mistretta et al., 2016). In these experiments, the voiding behavior was evaluated by cystometry measurements, but we used a frequency volume chart in metabolic cage experiments, which is noninvasive for animals and is both as reliable and reproducible as the cystometry measurements. Cold exposure induced a significant decrease in mean voided volume and an increase in voiding frequency and total voided volume (Table 3). In humans, it is known that the total voided volume increases because of the reduction of sweating in cold conditions, but in rats, the reason is unclear. As with the efficacy in cerebral infarcted rats, KPR-5714 increased the mean voided volume and decreased voiding frequency without affecting total voided volume in the cold exposure model (Table 3). It was suggested that KPR-5714 is useful for treating acute cold-induced OAB symptoms.

OAB is a chronic and sometimes debilitating condition of the LUT that negatively impacts the quality of life of millions of people worldwide (Abrams et al., 2002). In the clinical treatment of OAB, anticholinergic drugs and β3-adrenoceptor agonists have been widely used, but these drugs are considered to have potential side effects, such as dry mouth, constipation, or cardiovascular effects (Jayarajan and Radomski, 2013). Therefore, an unmet medical need for a pharmacological drug that can treat OAB patients with lesser side effects still remains.

The development of new therapeutic drugs with a different mode of action compared with current medicines has been

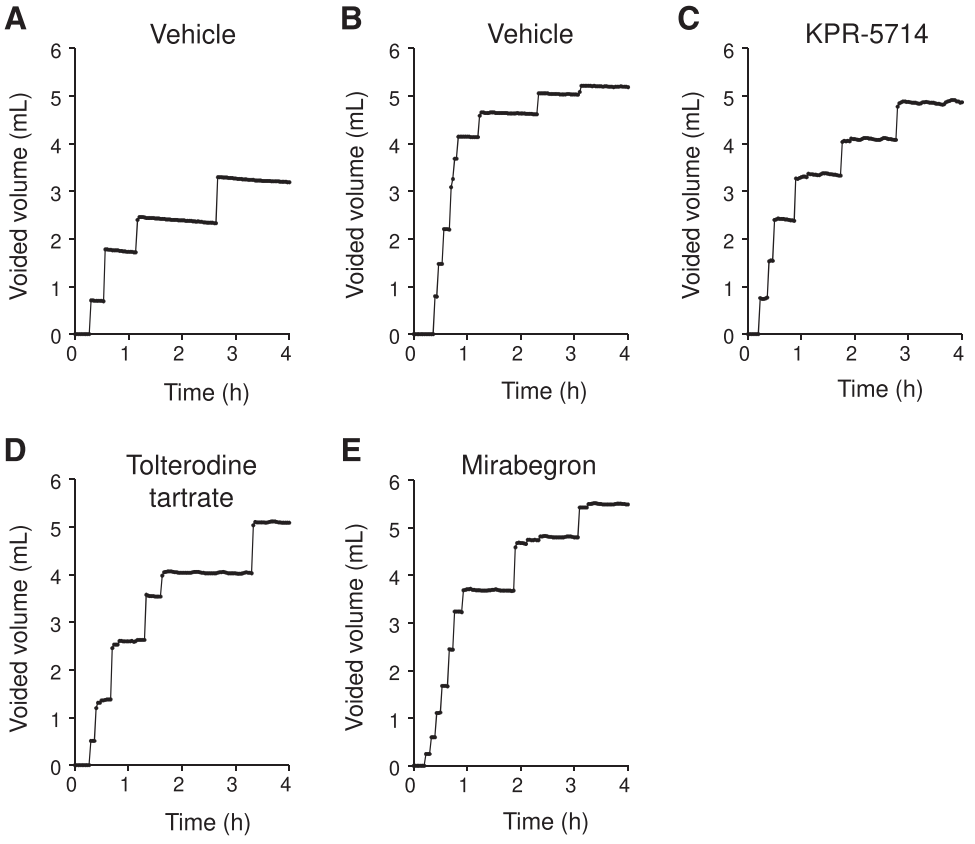


Fig. 7. Representative traces of frequency volume chart in rats given vehicle under the condition of room temperature (A) and rats given vehicle (B), KPR-5714 1 mg/kg (C), tolterodine tartrate 10 mg/kg (D), and mirabegron 10 mg/kg (E) under the condition of 4°C. Vehicle, KPR-5714, tolterodine tartrate, or mirabegron was orally administered.

TABLE 3

Effects of KPR-5714 on voiding behavior in rats exposed to cold

Data are presented as means ± S.E.M. Vehicle, KPR-5714, tolterodine tartrate, or mirabegron was orally administered. Mean voided volume, voiding frequency, and total voided volume after drug administration was evaluated during the metabolic cage experiments. ##$P < 0.01$ vs. vehicle-treated rats under the condition of room temperature, *$P < 0.05$; **$P < 0.01$ vs. vehicle-treated rats under the condition of 4°C.

Temperature condition	Compound	Dose (mg/kg)	n	Mean voided volume (ml)	Voiding frequency	Total voided volume (ml)
Room temperature (23–26°C)	Vehicle		10	1.05 ± 0.09	4.0 ± 0.6	3.89 ± 0.25
4°C	Vehicle		10	0.55 ± 0.05##	9.9 ± 0.7##	5.21 ± 0.28##
	KPR-5714	0.3	9	0.68 ± 0.04	7.9 ± 0.8	5.11 ± 0.28
		1	10	0.76 ± 0.06*	6.7 ± 0.5**	4.86 ± 0.10
4°C	Vehicle		10	0.57 ± 0.05##	10.1 ± 0.6##	5.57 ± 0.27##
	Tolterodine tartrate	10	10	0.74 ± 0.03**	7.2 ± 0.5**	5.23 ± 0.22
	Mirabegron	10	10	0.57 ± 0.04	9.0 ± 0.6	4.97 ± 0.30

[a] $P < 0.01$ vs. vehicle-treated rats under the condition of room temperature.
[b] $P < 0.05$.
[c] $P < 0.01$ vs. vehicle-treated rats under the condition of 4°C.

eagerly anticipated. Contribution of bladder afferent hyperactivity, especially the C-fiber, to the emergence of OAB symptoms has also been identified in clinical studies using neurotoxins such as botulinum toxin and resiniferatoxin. Therapies targeting TRPM8 expressed in C-fibers could be effective for reducing symptoms in OAB patients. KPR-5714 inhibited activation of bladder afferent hyperactivity and acted through a different molecular mechanism from anticholinergic drugs and β3-adrenoceptor agonists. In addition, KPR-5714 exhibited sufficient improvement in voiding behavior of rats with frequent urination when used as a monotherapy. Theoretically, it is supposed that a combination therapy (both KPR-5714 and an anticholinergic drug or a β3-adrenoceptor agonist) may uphold the potential of obtaining an additive effect compared with monotherapy.

In summary, KPR-5714 is a potent, selective, and orally available antagonist of TRPM8. KPR-5714 inhibited exaggerated activity of mechanosensitive bladder C-fiber, increased the mean voided volume, and decreased voiding frequency. In addition, improvement of KPR-5714 in frequent urination did not affect the total voided volume. Thus, KPR-5714 may provide a new therapeutic option for patients with hypersensitive bladder disorders, particularly in OAB.

Acknowledgments

We thank Hiroo Takeda, Satoshi Tatemichi, and Hiroki Kanbe for checking data and for helpful discussions.

Authorship Contributions

Participated in research design: Nakanishi, Fujimori, Aizawa, Hayashi, Igawa.

Conducted experiments: Nakanishi, Fujimori, Aizawa, Hayashi, Matsuzawa.

Contributed new reagents or analytic tools: Kobayashi, Hirasawa, Mutai, Tanada.

Performed data analysis: Nakanishi, Fujimori, Aizawa.

Wrote or contributed to the writing of the manuscript: Nakanishi, Fujimori, Aizawa, Igawa.

References

Abrams P, Cardozo L, Fall M, Griffiths D, Rosier P, Ulmsten U, van Kerrebroeck P, Victor A, and Wein A; Standardisation Sub-committee of the International Continence Society (2002) The standardisation of terminology of lower urinary tract function: report from the Standardisation Sub-committee of the International Continence Society. *Neurourol Urodyn* **21**:167–178.

Aizawa N, Fujimori Y, Kobayashi JI, Nakanishi O, Hirasawa H, Kume H, Homma Y, and Igawa Y (2018) KPR-2579, a novel TRPM8 antagonist, inhibits acetic acid-induced bladder afferent hyperactivity in rats. *Neurourol Urodyn* **37**:1633–1640.

Aizawa N, Ohshiro H, Watanabe S, Kume H, Homma Y, and Igawa Y (2019) RQ-00434739, a novel TRPM8 antagonist, inhibits prostaglandin E2-induced hyperactivity of the primary bladder afferent nerves in rats. *Life Sci* **218**:89–95.

Andersson KE (2019) TRP channels as lower urinary tract sensory targets. *Med Sci (Basel)* **7** Available from: 10.3390/medsci7050067.

Bautista DM, Siemens J, Glazer JM, Tsuruda PR, Basbaum AI, Stucky CL, Jordt SE, and Julius D (2007) The menthol receptor TRPM8 is the principal detector of environmental cold. *Nature* **448**:204–208.

Clapham DE (2003) TRP channels as cellular sensors. *Nature* **426**:517–524.

Edmondson SD, Zhu C, Kar NF, Di Salvo J, Nagabukuro H, Sacre-Salem B, Dingley K, Berger R, Goble SD, Morriello G, et al. (2016) Discovery of vibegron: a potent and selective β3 adrenergic receptor agonist for the treatment of overactive bladder. *J Med Chem* **59**:609–623.

Fowler CJ, Griffiths D, and de Groat WC (2008) The neural control of micturition. *Nat Rev Neurosci* **9**:453–466.

Ghei M and Malone-Lee J (2005) Using the circumstances of symptom experience to assess the severity of urgency in the overactive bladder. *J Urol* **174**:972–976.

González-Muñiz R, Bonache MA, Martín-Escura C, and Gómez-Monterrey I (2019) Recent progress in TRPM8 modulation: an update. *Int J Mol Sci* **20**:2618–2639.

Hatanaka T, Ukai M, Watanabe M, Someya A, Ohtake A, Suzuki M, Ueshima K, Sato S, and Sasamata M (2013) In vitro and in vivo pharmacological profile of the selective β3-adrenoceptor agonist mirabegron in rats. *Naunyn Schmiedebergs Arch Pharmacol* **386**:247–253.

Imamura T, Ishizuka O, Aizawa N, Zhong C, Ogawa T, Nakayama T, Tanabe T, and Nishizawa O (2008) Cold environmental stress induces detrusor overactivity via resiniferatoxin-sensitive nerves in conscious rats. *Neurourol Urodyn* **27**:348–352.

Jayarajan J and Radomski SB (2013) Pharmacotherapy of overactive bladder in adults: a review of efficacy, tolerability, and quality of life. *Res Rep Urol* **6**:1–16.

Kaidoh K, Igawa Y, Takeda H, Yamazaki Y, Akahane S, Miyata H, Ajisawa Y, Nishizawa O, and Andersson KE (2002) Effects of selective beta2 and beta3-adrenoceptor agonists on detrusor hyperreflexia in conscious cerebral infarcted rats. *J Urol* **168**:1247–1252.

Kanai A and Andersson KE (2010) Bladder afferent signaling: recent findings. *J Urol* **183**:1288–1295.

Kobayashi JI, Hirasawa H, Ozawa T, Ozawa T, Takeda H, Fujimori Y, Nakanishi O, Kamada N, and Ikeda T (2017) Synthesis and optimization of novel α-phenylglycinamides as selective TRPM8 antagonists. *Bioorg Med Chem* **25**: 727–742.

Kobayashi K, Fukuoka T, Obata K, Yamanaka H, Dai Y, Tokunaga A, and Noguchi K (2005) Distinct expression of TRPM8, TRPA1, and TRPV1 mRNAs in rat primary afferent neurons with adelta/c-fibers and colocalization with trk receptors. *J Comp Neurol* **493**:596–606.

Lashinger ES, Steiginga MS, Hieble JP, Leon LA, Gardner SD, Nagilla R, Davenport EA, Hoffman BE, Laping NJ, and Su X (2008) AMTB, a TRPM8 channel blocker: evidence in rats for activity in overactive bladder and painful bladder syndrome. *Am J Physiol Renal Physiol* **295**:F803–F810.

Lei Z, Ishizuka O, Imamura T, Noguchi W, Yamagishi T, Yokoyama H, Kurizaki Y, Sudha GS, Hosoda T, Nishizawa O, et al. (2013) Functional roles of transient receptor potential melastatin 8 (TRPM8) channels in the cold stress-induced detrusor overactivity pathways in conscious rats. *Neurourol Urodyn* **32**:500–504.

Longa EZ, Weinstein PR, Carlson S, and Cummins R (1989) Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke* **20**:84–91.

McKemy DD, Neuhausser WM, and Julius D (2002) Identification of a cold receptor reveals a general role for TRP channels in thermosensation. *Nature* **416**:52–58.

Mistretta FA, Russo A, Castiglione F, Bettiga A, Colciago G, Montorsi F, Brandolini L, Aramini A, Bianchini G, Allegretti M, et al. (2016) DFL23448, a novel transient receptor potential melastin 8-selective ion channel antagonist, modifies bladder function and reduces bladder overactivity in awake rats. *J Pharmacol Exp Ther* **356**:200–211.

Mukerji G, Yiangou Y, Corcoran SL, Selmer IS, Smith GD, Benham CD, Bountra C, Agarwal SK, and Anand P (2006) Cool and menthol receptor TRPM8 in human urinary bladder disorders and clinical correlations. *BMC Urol* **6**:6.

Peier AM, Moqrich A, Hergarden AC, Reeve AJ, Andersson DA, Story GM, Earley TJ, Dragoni I, McIntyre P, Bevan S, et al. (2002) A TRP channel that senses cold stimuli and menthol. *Cell* **108**:705–715.

Pérez de Vega MJ, Gómez-Monterrey I, Ferrer-Montiel A, and González-Muñiz R (2016) Transient receptor potential melastatin 8 channel (TRPM8) modulation: cool entryway for treating pain and cancer. *J Med Chem* **59**:10006–10029.

Skryma R, Prevarskaya N, Gkika D, and Shuba Y (2011) From urgency to frequency: facts and controversies of TRPs in the lower urinary tract. *Nat Rev Urol* **8**:617–630.

Suzuki M, Ohtake A, Yoshino T, Yuyama H, Hayashi A, Ukai M, Okutsu H, Noguchi Y, Sato S, and Sasamata M (2005) Effects of solifenacin succinate (YM905) on detrusor overactivity in conscious cerebral infarcted rats. *Eur J Pharmacol* **512**: 61–66.

Uvin P, Franken J, Pinto S, Rietjens R, Grammet L, Deruyver Y, Alpizar YA, Talavera K, Vennekens R, Everaerts W, et al. (2015) Essential role of transient receptor potential M8 (TRPM8) in a model of acute cold-induced urinary urgency. *Eur Urol* **68**:655–661.

Watanabe T, Maruyama S, Maruyama Y, Kageyama S, Shinbo H, Otsuka A, and Ozono S (2007) Seasonal changes in symptom score and uroflowmetry in patients with lower urinary tract symptoms. *Scand J Urol Nephrol* **41**: 521–526.

Woods M, Carson N, Norton NW, Sheldon JH, and Argentieri TM (2001) Efficacy of the beta3-adrenergic receptor agonist CL-316243 on experimental bladder hyperreflexia and detrusor instability in the rat. *J Urol* **166**:1142–1147.

Yokokawa R, Akino H, Ito H, Zha X, and Yokoyama O (2017) Nerve growth factor release from the urothelium increases via activation of bladder C-fiber in rats with cerebral infarction. *Neurourol Urodyn* **36**:1448–1455.

Yokoyama O, Ito H, Aoki Y, Oyama N, Miwa Y, and Akino H (2010) Selective α1A-blocker improves bladder storage function in rats via suppression of C-fiber afferent activity. *World J Urol* **28**:609–614.

Yoshimura N, Kaiho Y, Miyazato M, Yunoki T, Tai C, Chancellor MB, and Tyagi P (2008) Therapeutic receptor targets for lower urinary tract dysfunction. *Naunyn Schmiedebergs Arch Pharmacol* **377**:437–448.

Address correspondence to: Dr. Osamu Nakanishi, Central Research Laboratories, Kissei Pharmaceutical Co., Ltd., 4365-1, Kashiwabara, Hotaka, Azumino, Nagano 399-8304, Japan. E-mail: osamu_nakanishi@pharm.kissei.co.jp