KPR-5714, a Novel Transient Receptor Potential Melastatin 8 (TRPM8) Antagonist, Improves Overactive Bladder via Inhibition of Bladder Afferent Hyperactivity in Rats

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Running title page

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CV, conduction velocity

LUT, lower urinary tract

OAB, overactive bladder

SAAs, single-unit afferent activities

TRPM8, Transient Receptor Potential Melastatin 8

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ABSTRACT

Transient receptor potential 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 ([R]-3,3-difluoro-4-hydroxy-1-(2H-1,2,3-triazol-2-yl)butan-2-yl)-3-fluoro-2-[5-(4-fluorophenyl)-1H-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 non-selective cation channels that are activated by environmental and endogenous stimuli such as temperature, osmotic pressure, chemical substances, and mechanical stimulus (Clapham, 2003). Transient receptor potential 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 pathological 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
TRPM8 is involved in nociception and mechanosensory transduction in the LUT and its potential as a urological treatment target has been assessed (Skryma et al., 2011; Andersson, 2019). TRPM8-immunostaining is observed in human sub-urothelial 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 for 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 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-(2H-1,2,3-triazol-2-yl)butan-2-yl]-3-fluoro-2-[5-(4-fluorophenyl)-1H-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, AITC, capsaicin, RN-1747, N,N-dimethylacetamide, and acetic acid were obtained from Wako Pure Chemical Industries (Tokyo, Japan).

Molecular properties. Molecular weight, cLogP (calculated logarithm of octanol/water partition coefficient), tPSA (topological polar surface area) and LipE (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), 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, AITC, 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 a 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-h/12-h 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 $\geq 2.5$ 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$_2$O was achieved. SAAs were expressed as the firing rates (Hz) and evaluated in relation to intravesical pressure at each 5-cmH$_2$O interval. Average SAAs were totaled as a function of intravesical pressure. At the beginning of the experiments, recording was repeated 2 or 3 times consecutively at 5-min intervals to evaluate reproducibility. Subsequently, vehicle or KPR-5714 was intravenously administered. Then, 5 min after drug administration, the recording was repeated 3 times during 0.1% acetic acid instillation.
**Cystometry measurements.** Rats were anesthetized with urethane (1.2 g/kg intraperitoneally). 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 to 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-min intervals for 4 h. 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-min intervals for 4 h 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, San Diego, CA). Equality of variances were first analyzed with F-test or Bartlett’s test, 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 ASIC1a, ASIC3, Nav1.3, 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 (Fig. 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.
These changes in the group treated with KPR-5714 0.1 mg/kg were also significant for comparison with the vehicle-treatment 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 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 channels 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\textsubscript{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\textsubscript{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\textsubscript{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

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will be required, and this is a limitation of our present study.

We previously reported that TRPM8 may have physiological 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 (Fig. 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 rat 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 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 decreased also 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 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. (2016) 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). 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 was non-invasive for animals, and as both 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 to current medicines has been 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 act 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.
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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.
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receptor potential M8 (TRPM8) in a model of acute cold-induced urinary urgency.

*Eur Urol* **68**: 655-661.


FOOTNOTES

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FIGURES LEGENDS

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

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 (B: 0.01 mg/kg and C: 0.1 mg/kg) 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).

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, versus vehicle group.

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

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

IC₅₀ values of KPR-5714 for human and rat TRP channels.

<table>
<thead>
<tr>
<th>IC₅₀ value (nM)</th>
<th>Human TRPM8</th>
<th>Rat TRPM8</th>
<th>Human TRPA1</th>
<th>Human TRPV1</th>
<th>Human TRPV4</th>
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<tr>
<td>KPR-5714</td>
<td>25.3 ± 2.5ᵃ</td>
<td>22.4 ± 3.1ᵃ</td>
<td>&gt;10000ᵇ</td>
<td>&gt;10000ᵇ</td>
<td>&gt;10000ᶜ</td>
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</tbody>
</table>

ᵃ Data are presented as means ± S.E.M. from four experiments. ᵇ Data are presented from three experiments. ᶜ Data is obtained from one experiment.  Human or rat TRP channel expression plasmid was transfected into HEK293T cells.  Calcium influx assay was performed using transiently transfected cells, and the IC₅₀ values for each TRP channels were calculated.
## TABLE 2

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

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

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 versus vehicle-treated normal rats. * P < 0.05, ** P < 0.01 versus vehicle-treated cerebral infarcted rats.
TABLE 3

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

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

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.

## \( P < 0.01 \) versus vehicle-treated rats under the condition of room temperature, * \( P < 0.05 \), ** \( P < 0.01 \) versus vehicle-treated rats under the condition of 4°C.
Figure 1

mol. wt.: 474.42

$\text{cLogP} = 2.86$

$\text{tPSA} = 108.72$

$\text{LipE} = 4.74$
Figure 2

<table>
<thead>
<tr>
<th>(saline) baseline</th>
<th>(0.1% acetic acid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>after-1</td>
<td>after-2</td>
</tr>
<tr>
<td>after-3</td>
<td></td>
</tr>
</tbody>
</table>

A

- Intravesical Pressure (cmH2O)
- Firing Rate (Hz)
- Intravesical Pressure (cmH2O)

Vehicle i.v.

B

- Intravesical Pressure (cmH2O)
- Firing Rate (Hz)
- Intravesical Pressure (cmH2O)

KPR-5714
0.01 mg/kg, i.v.

C

- Intravesical Pressure (cmH2O)
- Firing Rate (Hz)
- Intravesical Pressure (cmH2O)

KPR-5714
0.1 mg/kg, i.v.

100 s
Figure 4

A

Intravesical Pressure (cmH₂O)

saline

0.25% acetic acid

B

Intravesical Pressure (cmH₂O)

Vehicle i.v.

0.25% acetic acid

C

Intravesical Pressure (cmH₂O)

KPR-5714
1 mg/kg, i.v.

0.25% acetic acid
Figure 5

(A) Intercontraction interval (% of baseline) and (B) Micturition pressure (% of baseline) for different doses of KPR-5714 (mg/kg) and Vehicle.
Figure 6

A. Vehicle

B. Vehicle

C. KPR-5714

D. Tolterodine tartrate

E. Mirabegron
Figure 7

A  Vehicle

B  Vehicle

C  KPR-5714

D  Tolterodine tartrate

E  Mirabegron