

JPET #214031

Title Page

Characteristics of TRK-130 (Naltalimide), a Novel Opioid Ligand, as a New
Therapeutic Agent for Overactive Bladder

Morihiro Fujimura, Naoki Izumimoto, Shinobu Momen, Satoru Yoshikawa, Ryosuke
Kobayashi, Sayoko Kanie, Mikito Hirakata, Toshikazu Komagata, Satoshi Okanishi,
Tadatoshi Hashimoto, Naoki Yoshimura and Koji Kawai

Pharmaceutical Research Laboratories, Toray Industries, Inc., Kanagawa Japan (M.F.,
N.I., S.M., S. Y., R.K., S.K., M.H., T.K., K.K.); Pharmaceutical Research Division,
Takeda Pharmaceutical Company Limited, Kanagawa Japan (S.O., T. H.), Departments
of Urology and Pharmacology & Chemical Biology, University of Pittsburgh, PA, USA
(N.Y).

JPET #214031

Running Title Page

a) Running Title

A new drug candidate for overactive bladder

b) Corresponding author

Morihiro Fujimura

Pharmaceutical Research Laboratories, Toray Industries Inc., 6-10-1 Teburo, Kamakura,

Kanagawa 248-8555 Japan.

Tel: +81-467-32-2111, Fax: +81-467-32-9862

E-mail: Morihiro_Fujimura@nts.toray.co.jp

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JPET #214031

TRK-130, *N*-[(5*R*, 6*R*, 14*S*)-17-(cyclopropylmethyl)-4, 5-epoxy-3, 14-dihydroxymorphinan-6-yl] phthalimide (Naltalimide); DAMGO, [D-Ala², N-Me⁴, Gly⁵-ol] enkephalin; DPDPE, cyclic [D-Pen², D-Pen⁵] enkephalin; U-69593, (+)-(5 α , 7 α , 8 β)-*N*-methyl-*N*-(7-(1-pyrrolidinyl)-1 oxaspiro [4, 5] dec-8-yl) benzeneacetamide; MOR, mu opioid receptor; DOR, delta opioid receptor; KOR, kappa opioid receptor; OAB, overactive bladder; CNS, central nervous system; PMC, pontine micturition center;

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JPET #214031

Abstract

We characterized TRK-130, *N*-[(5*R*, 6*R*, 14*S*)-17-(cyclopropylmethyl)-4,5-epoxy-3,14-dihydroxymorphinan-6-yl] phthalimide (naltalimide), an opioid ligand, to clarify the therapeutic potential for overactive bladder (OAB). In radioligand binding assays with cells expressing human μ -opioid receptors (MORs), δ -opioid receptors (DORs), or κ -opioid receptors (KORs), TRK-130 showed high selectivity for MORs (K_i for MORs, DORs, and KORs = 0.268, 121, and 8.97 nM, respectively). In a functional assay (cyclic AMP accumulation) with cells expressing each human opioid receptor subtype, TRK-130 showed potent but partial agonistic activity for MORs [EC_{50} (E_{max}) for MORs, DORs, and KORs = 2.39 nM (66.1%), 26.1 nM (71.0%), and 9.51 nM (62.6%), respectively]. In isovolumetric rhythmic bladder contractions (RBCs) in anesthetized guinea pigs, TRK-130 dose-dependently prolonged the shutdown time (the duration of complete cessation of the bladder contractions) (ED_{30} = 0.0034 mg/kg, i.v.) without affecting amplitude of RBCs. Furthermore, TRK-130 ameliorated formalin-induced frequent urination at doses of higher than 0.01 mg/kg, p.o. in guinea pigs under the freely moving condition. Meanwhile, TRK-130 showed only a negligible effect on the gastrointestinal transit at doses of up to 10 mg/kg, s.c. in mice. These results indicate that TRK-130 is a potent and selective human MOR partial

JPET #214031

agonist without undesirable opioid adverse effects such as constipation and enhance the storage function by suppressing the afferent limb of the micturition reflex pathway, suggesting that TRK-130 would be a new therapeutic agent for OAB.

JPET #214031

Introduction

Overactive bladder (OAB) is defined as “urinary urgency, usually accompanied by frequency and nocturia, with or without urgency urinary incontinence, in the absence of urinary tract infection or other obvious pathology” (Haylen et al., 2010). OAB is a distressing condition and one of the most prevalent complaints among both male and female adults. The prevalence of OAB is estimated to be 10%-20% of community residents in reports in the USA, Europe, and Japan (Stewart et al., 2003; Milsom et al., 2003; Homma et al., 2005). The prevalence of OAB increases as people age, and OAB is known to dramatically reduce people’s quality of life (QOL) to the extent that it makes long-distance travel and adequate sleep difficult (Liberian et al., 2001). The therapeutic approaches for OAB include behavior therapy, electrostimulation, and pharmacological therapy (Wein and Rackley, 2006; Wein, 2003; Cipullo et al., 2014). Although antimuscarinic drugs have been the current first-line pharmacotherapy for OAB, their persistence rates is low with less than 50% or 25% remaining on medication at 6 months or 1 year, respectively (Brostrøm and Hallas, 2009; Sexton et al., 2011; Chancellor et al., 2013). The high rate of discontinuing antimuscarinic drugs is considered to be attributable to the concurrence of many types of adverse effects such as dry mouth, constipation, blurred vision, voiding dysfunction, and tachycardia (Yarker et

JPET #214031

al., 1995; Andersson, 2004; Kelleher et al., 1997) as well as the lack of efficacy of antimuscarinic drugs in part due to the atropine-resistant contraction of the urinary bladder (Kelleher et al., 1997; Sahai et al., 2005; Sakakibara et al., 2011). Therefore, the development of new therapeutic agents with a mode of action different from that of antimuscarinic drugs has been eagerly anticipated. A number of clinical approaches to treat OAB with non-antimuscarinic mechanisms have proved to be beneficial, including β_3 -adrenergic receptor agonists (Nitti et al., 2013; Ohlstein et al., 2012). However, there is still no clinically proven pharmacotherapy for treating OAB that could act on the central nervous system (CNS), which controls lower urinary tract function. Moreover, recent advances in basic studies have revealed potential targets in the brain and spinal cord, including dopamine (Seki et al., 2001), γ -aminobutyric acid (GABA) (Morikawa et al., 1992), serotonin (Kakizaki et al., 2001), and opioid receptors (Soulard et al., 1992; Holt et al., 2005; Pehrson and Andersson, 2003; Pehrson et al., 2003; Pandita et al., 2003), etc. Thus, drugs acting on the CNS have been proposed as an alternative pharmacotherapy to treat OAB.

Opioids have been well known to exert an inhibitory effect on the micturition reflex at various CNS sites, including the pontine micturition center (PMC) (Noto et al., 1991), sacral parasympathetic nucleus (de Groat et al., 1983) and urethral sphincter

JPET #214031

motor nucleus in the spinal cord (Thor et al., 1989). Thus, opioid receptors are believed to be potential molecular targets for drugs acting on the CNS for OAB treatment. Although opioid receptor agonists such as morphine are known to inhibit the micturition reflex as demonstrated by experimental studies, they cannot be used as therapeutic agents for OAB owing to their adverse effects. Recently, some opioid receptor agonists that were without such undesirable opioid features but retained a voiding-suppressing action were demonstrated (Soulard et al., 1992; Holt et al., 2005; Pehrson and Andersson, 2003a, b; Pandita et al., 2003), suggesting the potential of opioid receptor agonists as a new therapeutic modality for OAB.

We have found the orally active morphinan derivative, TRK-130 (Fig. 1), as a potent and selective human μ -opioid receptor (MOR) partial agonist without undesirable opioid adverse effects such as constipation. Here, we report the *in vitro* profiles of TRK-130 and its effects on lower urinary tract function in guinea pigs.

JPET #214031

Materials and Methods

Drugs. TRK-130 was synthesized at Pharmaceutical Research Laboratories, Toray Industries, Inc. (Kanagawa, Japan) according to a procedure, for example, described in a previous literature (Simon et al., 1994). Morphine was obtained from Takeda Pharmaceutical Company Limited (Osaka, Japan). DAMGO, DPDPE, U-69593, naloxone, forskolin, and oxybutynin were obtained from Sigma-Aldrich Co., LLC. (St. Louis, MO). Buprenorphine (Lepetan Injection[®]) was obtained from Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan). [³H]-diprenorphine and [³H]-naltrindole were obtained from PerkinElmer, Inc. (Waltham, MA).

Radioligand Binding. The radioligand binding studies were conducted by Eurofins Panlabs Taiwan, Ltd. (Taipei, Taiwan) (Catalog #260110, 260210 and 260410). Human MOR and δ -opioid receptor (DOR) binding assays were performed using a membrane preparation derived from CHO cells stably expressing human MORs or DORs. Human κ -opioid receptor (KOR) binding assay was performed using a membrane preparation derived from HEK-293 cells stably expressing human KORs. A typical incubation mixture in a test tube for MOR and KOR consisted of 200 μ L of membrane suspension in 50 mM Tris-HCl buffer (pH 7.4), 2.2 μ L of a work solution of these drugs, and 20 μ L of [³H]-diprenorphine solution in 50 mM Tris-HCl buffer (pH

JPET #214031

7.4) (a final concentration of 0.6 nM). A typical incubation mixture in a test tube for DOR consisted of 200 μ L of membrane suspension in 50 mM Tris-HCl buffer (pH 7.4), containing 5 mM MgCl₂, 2.2 μ L of a work solution of these drugs, and 20 μ L of [³H]-naltrindole solution in 50 mM Tris-HCl buffer (pH 7.4), containing 5 mM MgCl₂ (a final concentration of 0.9 nM). Incubation was performed at 25°C for 60 minutes for MOR and KOR and 120 minutes for DOR and terminated by filtration through a GF/B filter. The radioactivity trapped on the filter was determined using the LKB Betaplate Scintillation Counter to calculate the radioactivity bound to the receptors. Nonspecific binding was determined as the radioligand bound in the presence of 10 μ M naloxone.

Cell Culture. CHO-K1 cells (host cells) were transfected with the cDNA for human MORs and KORs (from the amygdala or thalamus, cDNA Library, Clontech Laboratories, Inc., Mountain View, CA) in the pEF/myc/cyto vector, and for human KORs in the pCR3 vector by the Lipofectamine transfection method. CHO-dhfr(-) cells (host cells) were transfected with the cDNA for human DORs (from the SK-N-SH cells) in the pCR3 vector by the Lipofectamine transfection method. Stably transfected clone was selected and their expression were confirmed by radioligand binding assay with their selective ligands, [³H]-DAMGO, [³H]-U69593 and [³H]-DPDPE (PerkinElmer, Inc.), respectively. The CHO-K1 cells and CHO-dhfr(-)

JPET #214031

cells, including those expressing human opioid receptors, were grown and maintained basically in α -MEM with or without ribonucleosides and deoxyribonucleosides, respectively, in the presence of 10% FBS, 100 units/mL penicillin, 100 μ g/mL streptomycin, and 0.6 mg/mL G418 disulfate in 5% CO₂ at 37°C. After CHO-dhfr(-) and CHO-K1 cells were grown, the cells were rinsed with PBS(-) twice and then incubated in 0.53 mM EDTA/PBS at room temperature until the cells were detached from culture flask. The cells were collected by centrifugation and suspended in HBSS containing 0.5 mM IBMX, 5 mM HEPES, and 0.1% BSA (pH 7.4) (hereinafter referred to as stimulation buffer) to give a final concentration of 10,000 cells/ μ L. This suspension was used in the forskolin-stimulated cyclic AMP (cAMP) accumulation assay detailed below.

cAMP Accumulation. The assay of forskolin-stimulated cAMP accumulation was performed using an AlphaScreen cAMP Assay Kit (PerkinElmer, Inc.) according to the manufacture's instruction. In brief, a typical incubation mixture was prepared by the addition of 5 μ L of a suspension containing CHO cells (7,500-10,000 cells) and anti-cAMP acceptor beads (1 unit) in stimulation buffer to 5 μ L of the drug solution (a prescribed final concentration) in stimulation buffer containing 100 μ M forskolin. Incubation was performed in the dark at 25°C for 60 minutes. At the end of the

JPET #214031

incubation, 15 μ L of a conditioned biotinylated-cAMP detection solution containing streptavidin donor beads was added to the incubation mixture (1 unit/well). The incubation mixture was further incubated in the dark at 25°C for 120 minutes. cAMP accumulation was quantitated as AlphaScreen signals using a Fusion- α Universal Microplate Reader (PerkinElmer, Inc.). As a stock solution, TRK-130 was dissolved in 10% dimethyl sulfoxide solution containing methanesulfonic acid (1.1 molar equivalents to TRK-130); DAMGO, DPDPE, and morphine were dissolved in distilled water and U-69593 in ethanol. Each stock solution was serially diluted with stimulation buffer for use.

Distension-induced Isovolumetric Rhythmic Bladder Contractions (RBCs).

This experiment was conducted as described previously, with some modification (Doi et al., 2000). Female Hartley guinea pigs (Japan SLC, Inc., weighing 265 to 320 g) were anesthetized with an intraperitoneal injection of urethane (1.2 g/kg). The urinary bladder was exposed through a midline incision of the abdomen, and the urethra was ligated. A polyethylene tube (PE-100) was inserted into the bladder dome for recording the intravesical pressure and another for injection of saline into the bladder. Physiological saline was injected with increments of each 0.2 ml into the bladder, and the injection was stopped when continuous isovolumetric RBCs appeared in order to

JPET #214031

evaluate the effects of drug treatment under the threshold volume condition (Doi et al., 2000). Drugs were intravenously administered to the animals showing stable RBCs. Following drug administration, the intravesical pressure was recorded until the RBC reappeared with a cut-off time of 60 minutes. The parameters measured were the shutdown time (the duration of complete cessation of the bladder contractions) before and after drug administration and the maximum intravesical pressures at contractions immediately before and after drug administration. Drugs were dissolved in 5% xylitol solution (Otsuka Pharmaceutical Co., Ltd.) containing 0.02 % citric acid and injected at a volume of 0.5 mL/kg.

Chemically Induced Pollakiuria Model. Two days before the experiment, female Hartley guinea pigs (Japan SLC, Inc., weighing 280 to 353 g) received an intravesical instillation of formalin. Under ether anesthesia, a 4 Fr. Groshong[®] catheter (Bard Access Systems, Inc., Salt Lake City, UT) was placed transurethrally. The tip of the catheter was positioned in the bladder. Urine was drained from the bladder, and 2.5% formalin saline solution (1 mL) was instilled into the bladder through the catheter for 1 minute, and then the solution was drained off. Animals with intravesical instillation of saline instead of formalin saline served as the saline-instilled control. A metabolic cage mounted on an electrical balance was used for measuring spontaneous

JPET #214031

voiding. Between the cage and the balance, a wire mesh was inserted to catch the feces. The balance was connected to a computer, and the digital output values of the balance were stored. A change in the output values of the balance was regarded as a voiding episode. After completion of the experiment, the sequential data obtained from the balance were analyzed to obtain the voiding parameters. On the day of the experiment, following a 30-minute acclimation period after placing the animals in the cages, water was given to the animals at a volume of 40 mL/kg. Following a 3-hour voiding measurement period after water loading (the predrug control session), the animals were subjected to oral administration with vehicle or TRK-130, followed by additional oral water loading at a volume of 40 mL/kg. The voided volume was then measured for another 3 hours (the drug session). The percentage of the voiding parameters (the number of voiding episodes, mean urine volume per void, and total urine volume) obtained during the drug session to those obtained during the predrug control session was calculated and used to evaluate the drug effect. TRK-130 was dissolved in 5% xylitol solution (Otsuka Pharmaceutical Co., Ltd.) containing 0.02% citric acid and injected at a volume of 2 mL/kg.

Gastrointestinal Transit. Male ddY mice (Japan SLC, Inc., weighing 22.8 to 27.8 g) were made to fast on the day before use in the gastrointestinal transit experiment.

JPET #214031

This experiment was performed using a single-blinded study protocol. In the morning on the day of the experiment, drugs were subcutaneously administered to the animals. Fifteen minutes after the administration, the animals were orally administered 5% gum arabic containing 10% charcoal (charcoal meal) at a dosing volume of 0.25 mL/body. Twenty minutes after the administration of the charcoal meal, the animals were euthanized by cervical dislocation. The small intestine from the pylorus to the cecum was removed from the body. The isolated intestine was straightened to measure its total length (cm) and the farthest distance (cm), which the charcoal meal traveled in the intestine. TRK-130 was dissolved in 5% xylitol containing 0.1% citric acid, and morphine and buprenorphine were dissolved in physiological saline and injected at a volume of 10 mL/kg.

Calculations and Statistics. Results were expressed as mean \pm SEM or mean with 95% confidence intervals.

For the receptor binding assay, the K_i values were calculated from the equation, $K_i = IC_{50} / (1 + [Radioligand] / K_d)$. The IC_{50} value was determined using nonlinear logistic regression analysis. For the cAMP accumulation assay, the EC_{50} values were determined using nonlinear optimization. For comparison of the E_{max} values (the percentage to maximum inhibition of cAMP accumulation attained by the full agonist at

JPET #214031

the corresponding receptor subtypes), a multiple comparison was performed using the two-tailed Tukey test or two-tailed Steel-Dwass test. For RBCs, The ED₃₀ values were determined from the percentage of maximum possible effects (%MPE = {(shutdown time in the drug-treated group - shutdown time in the vehicle-treated group) / [cut-off time (60 minutes) - shutdown time in the vehicle-treated group]} x 100) using a nonlinear logistic regression analysis. The cut-off time value of 60 minutes was used as the shutdown time when the disappearance of RBCs after the drug treatment lasted for longer than 60 minutes (cut-off time). The postdrug maximum intravesical pressure was converted into the percentage of the predrug value and used for analysis. The differences between the groups were analyzed using the Student's t-test, Aspin-Welch test, one-tailed Williams' test, or one-tailed Shirley-Williams' test. For the pollakiuria model, the differences in the voiding parameters between the saline-instilled control group and the formalin-instilled vehicle-treated group were analyzed using the Aspin-Welch test. The differences in the voiding parameters between the formalin-instilled vehicle-treated control group and the formalin-instilled TRK-130-treated groups were analyzed using the one-tailed Williams' test or one-tailed Shirley-Williams' test. For the gastrointestinal transit, ID₅₀ values were determined from the relative transit rates in percentage to the transit rate in the vehicle control

JPET #214031

group using the least square linear regression. The differences in the gastrointestinal transit rate between the groups were analyzed using the Williams' test or Shirley-Williams' test.

Ethical Consideration. All the animal experiments in this study were approved by the Animal Ethics Committee of the Research and Development Division, Toray or Experimental Animal Care and Use Committee of Takeda.

JPET #214031

Results

Binding Affinities of TRK-130 for Human MOR, DOR, and KOR. In radioligand binding assays, TRK-130 demonstrated selectivity for MOR over DOR and KOR (Table 1) with the K_i (affinity) values of 0.268 nM for human MOR, 121 nM for human DOR, and 8.97 nM for human KOR. The K_i values of morphine were 11.5 nM for human MOR, 1517 nM for human DOR, and 445 nM for human KOR.

Agonist Activities of TRK-130 for Human MOR, DOR, and KOR. In the experiment, DAMGO, DPDPE, and U-69593 were used as standard full agonists for human MOR, DOR, and KOR receptors, respectively.

In cells expressing human MORs, TRK-130 showed a concentration-dependent inhibition of intracellular cAMP accumulation induced by stimulation with forskolin (50 μ M) (Fig. 2). The E_{max} (efficacy) and EC_{50} values (potency) were 66.1% and 2.39 nM, respectively (Table 2). Morphine and buprenorphine also showed a concentration-dependent inhibition of intracellular cAMP accumulation, with the E_{max} values of 100.0% and 90.8% and the EC_{50} values of 19.9 nM and 4.49 nM, respectively (Fig. 2, Table 2). In cells expressing human DORs and KORs, TRK-130 also concentration-dependently inhibited intracellular cAMP accumulation (Fig. 2). The E_{max} and EC_{50} values were 71.0% and 26.1 nM for DOR, and 62.6% and 9.51 nM for

JPET #214031

KOR, respectively (Table 2). The E_{\max} and EC_{50} values of buprenorphine were 62.2% and 8.00 nM for DOR, and 20.5% and 2.08 nM for KOR, respectively (Fig. 2, Table 2). Because the inhibition effect of morphine did not reach its peak in the concentration range tested, its E_{\max} and EC_{50} values were not calculated in cells expressing DORs or KORs (Fig. 2, Table 2).

Effects of TRK-130 on RBCs induced by bladder distension. Typical tracings of distension-induced RBCs in female guinea pigs and the effects of intravenous TRK-130 and oxybutynin at doses of 0.01 mg/kg and 1 mg/kg, respectively, are shown in Fig. 3. The frequency of distension-induced RBCs is believed to be regulated by the micturition center in the CNS (Maggi et al., 1986). Intravenous administration with TRK-130 (0.00125-0.01 mg/kg) dose-dependently prolonged the shutdown time with an ED_{30} value of 0.0034 mg/kg (Table 3) without affecting the maximum intravesical pressure (Table 4). Intravenous administration with morphine (0.25-1 mg/kg) also dose-dependently prolonged the shutdown time with an ED_{30} value of 0.62 mg/kg (Table 3) without affecting the maximum intravesical pressure (Table 4). In contrast, intravenous administration with oxybutynin at a dose of 1 mg/kg significantly attenuated the maximum intravesical pressure (Table 4), although it did not show any effect on the generation of contractions (Table 3).

JPET #214031

Effects of TRK-130 on Chemically Induced Pollakiuria Model. The effects of oral administration with TRK-130 (0.003-0.03 mg/kg) on frequent urination (a status of increased number of voiding episodes and reduced urine volume per void) of conscious, freely moving female guinea pigs with formalin-induced pollakiuria are shown in Fig. 4. Administration with TRK-130 dose-dependently reduced the number of voiding episodes without affecting total urine production (data not shown), and the minimal effective dose was 0.01 mg/kg for either index (the number of voiding episodes and mean urine volume per void).

Effects of TRK-130 on Gastrointestinal Transit. Comparison with the vehicle control group in terms of the gastrointestinal transit rate indicated no significant changes in the group subcutaneously treated with TRK-130 even at 10 mg/kg (Fig. 5). In contrast, comparison with the vehicle control group showed significant inhibition of the gastrointestinal transit in the group subcutaneously treated with morphine at any of the examined doses (0.3-10 mg/kg) (Fig. 5). Similarly, comparison with the vehicle control group showed significant inhibition of the gastrointestinal transit in the group subcutaneously treated with buprenorphine at any of the examined doses (0.03-1 mg/kg) (Fig. 5). Morphine showed approximately 90% inhibitions at maximum whereas buprenorphine showed a plateau of approximately 50% inhibitions. From the relative

JPET #214031

gastrointestinal transit rates, the ID_{50} values of morphine and buprenorphine were estimated to be 1.01 and 0.326 mg/kg, respectively (Table 5). As for TRK-130, no ID_{50} value was calculated, because TRK-130 showed a less than 50% inhibition of the gastrointestinal transit rate at any of the doses examined.

JPET #214031

Discussion

A series of the current studies was undertaken to characterize the pharmacological profile of TRK-130 *in vitro* and *in vivo*. To examine the affinities to MOR, DOR, and KOR, TRK-130 and morphine were tested at 5-7 concentrations to estimate their binding inhibition constants (the K_i values). Comparison of the estimated K_i values indicated that TRK-130 had a higher affinity to any of the opioid receptor examined than morphine. In addition, both TRK-130 and morphine showed affinities to these different receptor subtypes in the order of $\mu > \kappa \gg \delta$. In the cAMP accumulation assay of MOR, the E_{\max} value of TRK-130 and buprenorphine, a typical MOR partial agonist (Huang et al., 2001; Selley et al., 1998), were 66.1% and 90.8%, respectively, which are significantly lower values compared with those of DAMGO, a standard MOR full agonist. This finding suggests that TRK-130 is a MOR partial agonist, similar to buprenorphine. The E_{\max} value of morphine was 100.0%, and the E_{\max} value of TRK-130 was also significantly lower compared with that of morphine and buprenorphine. The EC_{50} value for MOR was low and in the order of DAMGO, TRK-130, buprenorphine, and morphine. It implies that potencies to human MOR were in the order of DAMGO > TRK-130 > buprenorphine \gg morphine.

In cells expressing human DOR and KOR, the E_{\max} values of TRK-130 were

JPET #214031

significantly lower compared with those of DPDPE and U-69593, standard full agonists, respectively. This finding suggests that TRK-130 is also a DOR and KOR partial agonist. Each standard full agonist at the corresponding receptor subtypes induced no agonist activity in host cells. Taken together, these results demonstrate TRK-130 to be a potent and selective human MOR partial agonist.

Regarding RBCs induced by bladder distention, TRK-130 and morphine suppress the micturition reflex without affecting the contractile force of the detrusor, while oxybutynin attenuates the contractile force without affecting the generation of the voiding reflex in the same animal model. The current result of the effect of oxybutynin on RBCs is consistent with previous reports (Shimizu et al., 2001; Doi et al., 2000). In addition, we have performed an *in vitro* study and found that TRK-130 at 1 μ M did not significantly inhibit electrical field stimulation-induced contractions in isolated rat bladder strips (data not shown). This concentration (1 μ M) is considerably high compared to the plasma concentration (C_{5min}) of 11.85 ng/mL (approximately 25 nM) after administration of TRK-130 at a dose of 0.1 mg/kg (i.v.), which is 10-fold greater than the highest dose (0.01 mg/kg) used in the present *in vivo* study. These results suggest that the site of action of TRK-130 lies in the afferent limb of micturition reflex pathway. Furthermore, TRK-130 exerts a more prominent suppressing effect on

JPET #214031

the micturition reflex than morphine because the effects of TRK-130 occurred at substantially lower doses than morphine. This may reflect the greater affinity and potency of TRK-130 for MOR. Taken together, these results strongly support the assumption that TRK-130 may enhance the storage function by modulating the afferent limb of the micturition reflex, in contrast to oxybutynin acting on the bladder efferent function and/or bladder contractility.

The intravesical instillation of formalin significantly increased voiding episodes compared with the instillation of vehicle and significantly decreased the mean urine volume per void. Compared with vehicle, oral administration with TRK-130 dose-dependently attenuated the effects of formalin on voiding parameters. TRK-130 at doses of 0.01 and 0.03 mg/kg significantly reduced voiding episodes and significantly increased urine volume per void, which was expressed as a percentage of the predrug values, in a conscious, freely moving animal model of pollakiuria induced by intravesical formalin administration. The effects of tramadol, which is well known to be a MOR agonist and an inhibitor of 5-hydroxytryptamine and noradrenaline reuptake, on micturition in normal and urinary frequent, conscious rats have been reported previously (Pehrson and Andersson, 2003a, b; Pandita et al., 2003). Tramadol effectively inhibits micturition without decreasing the micturition pressure by mainly

JPET #214031

stimulating MORs. At the same time, tramadol is reported to have a diuretic effect by activating on KORs. Regarding diuresis, TRK-130 dose-dependently reduced frequent urination without affecting diuresis (data not shown). Furthermore, tramadol suppressed bladder pain-related behaviors in mice with chemically induced cystitis (Oyama et al., 2012). In the current study, we did not evaluate the effect of TRK-130 on chemically-induced bladder pain. Thus, further investigations are needed to assess the potential of TRK-130 to relieve bladder pain. Taken together, these *in vivo* studies conducted in conscious and anesthetized guinea pigs indicate that TRK-130 has a favorable profile for the treatment of OAB.

It is well known that endogenous opioids are involved in the modulation of the micturition reflex mainly by the stimulation of MORs and DORs. The tonic enkephalinergic inhibition of the micturition reflex is believed to be mediated at several possible levels including the peripheral bladder ganglia, sacral spinal cord, or brainstem (Noto et al., 1991). Studies using several types of MOR drugs have shown that the MOR modulation of bladder motility is exerted both at supraspinal and spinal sites in various species. Microinjection of the MOR agonist, DAMGO, into the ventrolateral periaqueductal gray in rats (Matsumoto et al., 2004) or fentanyl into the PMC in cats (Noto et al., 1991) inhibited reflex micturition. The intrathecal or

JPET #214031

intracerebroventricular administration of morphine also inhibited bladder motility, which was abolished by naloxone in rats (Dray and Metsch, 1984; Dray and Nunan, 1985). These previous findings suggest that MOR is a potential pharmacological target for OAB treatment by acting mainly on the afferent limb of the micturition reflex.

Although MOR agonists such as morphine are potential pharmacological drugs, they cannot be used as therapeutic agents for OAB due to adverse effects. In the current study, TRK-130 was tested for potential adverse effects on the gastrointestinal transit, which are typically shown with MOR agonists. TRK-130 had a negligible effect on the gastrointestinal transit at doses at up to 10 mg/kg, when compared with the effects of morphine or buprenorphine. This result sets TRK-130 apart from the conventional MOR agonists.

In conclusion, we demonstrated that the orally active morphinan derivative, TRK-130, is a potent and selective human MOR partial agonist without undesirable opioid-related adverse effects such as constipation and enhances the bladder storage function by modulating the afferent limb of the micturition reflex pathway *in vivo*. These findings suggest that TRK-130 would be a new therapeutic agent for OAB treatment and potentially have a better pharmacological profile than antimuscarinic drugs.

JPET #214031

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JPET #214031

Authorship Contributions

Participated in research design: Morihiko, I. Naoki, Sayoko, Tadatoshi, and Y. Naoki.

Conducted experiments: Morihiko, I. Naoki, Shinobu, Ryosuke, and Satoshi.

Contributed new reagents or analytic tools: Tadatoshi.

Performed data analysis: Morihiko, I. Naoki, Shinobu, Ryosuke, Sayoko, Satoshi, and Tadatoshi.

Wrote or contributed to the writing of the manuscripts: Morihiko, Satoru, Mikito, Toshikazu, Koji, Tadatoshi, and Y Naoki.

JPET #214031

References

- Andersson KE (2004) Antimuscarinics for treatment of overactive bladder. *Lancet Neurol* 3: 46-53.
- Brostrøm S and Hallas J (2009) Persistence of antimuscarinic drug use. *Eur J Clin Pharmacol* 65: 309-314.
- Cipullo LMA, Cosimato C, Filippelli A, Conti V, Izzo V, Zullo F, and Guida M (2014) Pharmacological approach to overactive bladder and urge urinary incontinence in women: an overview. *Eur J Obstet Gynecol Reprod Biol* 174: 27-34.
- Chancellor MB, Migliaccio-Walle K, Bramley TJ, Chaudhari SL, Corbell C, and Globe D (2013) Long-term patterns of use and treatment failure with anticholinergic agents for overactive bladder. *Clin Ther* 35: 1744-1751.
- de Groat WC, Kawatani M, Hisamitsu T, Lowe I, Morgan C, Roppolo J, Booth AM, Nadelhaft I, Kuo D, and Thor K (1983) The role of neuropeptides in the sacral autonomic reflex pathways of the cat. *J Auton Nerv Syst* 7: 339-350.
- Doi T, Kamo I, Imai S, Okanishi S, Ikeura Y, and Natsugari H (2000) Effects of TAK-637, a tachykinin receptor antagonist, on the micturition reflex in guinea pigs. *Eur J Pharmacol* 395: 241-246.
- Dray A and Metsch R (1984) Opioid receptor subtypes involved in the central inhibition of urinary bladder motility. *Eur J Pharmacol* 104: 47-53.
- Dray A and Nunan L (1985) Opioid inhibition of reflex urinary bladder contractions: dissociation of supraspinal and spinal mechanisms. *Brain Res* 337: 142-145.
- Haylen BT, de Ridder D, Freeman RM, Swift SE, Berghmans B, Lee J, Monga A, Petri E, Rizk DE, Sand PK, and Schaer GN (2010) An International Urogynecological

JPET #214031

- Association (IUGA) / International Continence Society (ICS) joint report on the terminology for female pelvic floor dysfunction. *Neurourol Urodyn* 29: 4-20.
- Holt JDS, Watson MJ, Chang JP, O'Neill SJ, Wei K, Pendergast W, Gengo PJ, and Chang KJ (2005) DPI-221 [4-((α -S)- α -((2S, 5R)-2, 5-Dimethyl-4-(3-fluorobenzyl)-1-piperazinyl)benzyl)-N, N-diethylbenzamide]: A novel nonpeptide δ receptor agonist producing increased micturition interval in normal rats. *J Pharmacol Exp Ther* 315: 601-608.
- Homma Y, Yamaguchi O, Hayashi K, and the members of the neurogenic bladder society committee (2005) An epidemiological survey of overactive bladder symptoms in Japan. *BJU Int* 96: 1314-1318.
- Huang P, Kehner GB, Cowan A, and Liu-chen L (2001) Comparison of pharmacological activities of buprenorphine and norbuprenorphine: norbuprenorphine is a potent opioid agonist. *J Pharmacol Exp Ther* 297: 688-695.
- Kakizaki H, Yoshiyama M, Koyanagi T, and de Groat W (2001) Effects of WAY100635, a selective 5-HT_{1A}-receptor antagonist on the micturition-reflex pathway in the rat. *Am J Physiol Regulatory Integrative Comp Physiol* 280: R1407-R1413.
- Kelleher CJ, Cardozo LD, Khullar V, and Salvatore S (1997) A medium-term analysis of the subjective efficacy of treatment for women with detrusor instability and low bladder compliance. *Br J Obstet Gynaecol* 104: 988-993.
- Lieberman JN, Hunt TL, Stewart WF, Wein A, Zhou Z, Herzog AR, Lipton RB, and Diokno AC (2001) Health-related quality of life among adults with symptoms of

JPET #214031

- overactive bladder: Results from a U.S. community-based survey. *Urology* 57: 1044-1050.
- Maggi CA, Santicioli P, and Meli A (1986) Somatovesical and vesicovesical excitatory reflexes in urethane-anaesthetized rats. *Brain Res* 380: 83-93.
- Matsumoto S, Levendusky MC, Longhurst PA, Levin RM, and Millington WR (2004) Activation of mu opioid receptors in the ventrolateral periaqueductal gray inhibits reflex micturition in anesthetized rats. *Neurosci Lett* 363: 116-119.
- Milsom I, Abrams P, Cardozo L, Roberts RG, Thuroff J, and Wein AJ (2001) How widespread are the symptoms of an overactive bladder and how are they managed? A population-based prevalence study. *BJU Int* 87: 760-766.
- Morikawa K, Hashimoto S, Yamauchi T, Kato H, Ito Y, and Gomi Y (1992) Inhibitory effect of inaperisone hydrochloride (inaperisone), a new centrally acting muscle relaxant, on the micturition reflex. *Eur J Pharmacol* 213: 409-415.
- Nitti VW, Khullar V, van Kerrebroeck P, Herschorn S, Cambroner J, Angulo JC, Blauwet MB, Dorrepaal C, Siddiqui E, and Martin NE (2013) Mirabegron for the treatment of overactive bladder: a prespecified pooled efficacy analysis and pooled safety analysis of three randomised, double-blind, placebo-controlled, phase III studies. *Int J Clin Pract* 67: 619-632.
- Noto H, Roppolo JR, de Groat WC, Nishizawa O, Sugaya K, and Tsuchida S (1991) Opioid modulation of the micturition reflex at the level of the pontine micturition center. *Urol Int* 47(supple 1): 19-22.

JPET #214031

- Ohlstein EH, von Keitz A, and Michel MC (2012) A multicenter, double-blind, randomized, placebo-controlled trial of the β_3 -adrenoceptor agonist solabegron for overactive bladder. *Eur Urol* 62: 834-840.
- Oyama T, Homan T, Kyotani J, and Oka M (2012) Effect of tramadol on pain-related behaviors and bladder overactivity in rodent cystitis models. *Eur J Pharmacol* 676: 75-80.
- Pandita RK, Pehrson R, Christoph T, Friderichs E, and Andersson KE (2003) Actions of tramadol on micturition in awake, freely moving rats. *Br J Pharmacol* 139: 741-748.
- Pehrson R and Andersson KE (2003) Tramadol inhibits rat detrusor overactivity caused by dopamine receptor stimulation. *J Urol* 170: 272-275.
- Pehrson R, Stenman E, and Andersson KE (2003) Effects of tramadol on rat detrusor overactivity induced by experimental cerebral infarction. *Eur Urol* 44: 495-499.
- Sahai A, Khan M, Fowler CJ, and Dasgupta P (2005) Botulinum toxin for the treatment of lower urinary tract symptoms: a review. *Neurourol Urodyn* 24: 2-12.
- Sakakibara F, Kiniwa M, and Nanri M (2011) Are antimuscarinic drugs effective against urinary frequency mediated by atropine-resistant contractions? *J Pharmacol Sci* 115: 364-373.
- Seki S, Igawa Y, Kaidoh K, Ishizuka O, Nishizawa O, and Andersson KE (2001) Role of dopamine D₁ and D₂ receptors in the micturition reflex in conscious rats. *Neurourol Urodyn* 20: 105-113.

JPET #214031

- Selley DE, Liu Q, and Childers SR (1998) Signal transduction correlates of mu opioid agonist intrinsic efficacy: receptor-stimulated [³⁵S]GTPγS binding in mMOR-CHO cells and rat thalamus. *J Pharmacol Exp Ther* 285: 496–505.
- Sexton CC, Notte SM, Maroulis C, Dmochowski RR, Cardozo L, Subramanian D, and Coyne KS (2011) Persistence and adherence in the treatment of overactive bladder syndrome with anticholinergic therapy: a systematic review of the literature. *Int J Clin Pract* 65: 567-585.
- Shimizu I, Kawashima K, Ishii D, Oku S, Kohayakawa H, and Oka M (2001) Pharmacological actions of AH-9700 on micturition reflex in anesthetized rats. *Eur J Pharmacol* 412: 171-179.
- Simon C, Hosztafi S, and Makleit S (1994) Stereoselective synthesis of β-naltrexol, β-naloxol, β-naloxamine, β-naltrexamine and related compounds by the application of the Mitsunobu reaction. *Tetrahedron* 50: 9757-9768.
- Soulard C, Pascaud X, Roman FJ, Grouhel A, and Junien JL (1992) Pharmacological evaluation of JO 1870: relation to the potential treatment of urinary bladder incontinence. *J Pharmacol Exp Ther* 260: 1152-1158.
- Stewart WF, van Rooyen JB, Cundiff GW, Abrams P, Herzog AR, Corey R, Hunt TL, and Wein AJ (2003) Prevalence and burden of overactive bladder in the United States. *World J Urol* 20: 327-336.
- Thor KB, Hisamitsu T, Roppolo JR, Tuttle P, Nagel J, and de Groat WC (1989) Selective inhibitory effects of ethylketocyclazocine on reflex pathways to the external urethral sphincter of the cat. *J Pharmacol Exp Ther* 248: 1018-1025.

JPET #214031

Wein AJ (2003) Diagnosis and treatment of the overactive bladder. *Urology* 62(Supple 5B): 20-27.

Wein AJ and Rackley RR (2006) Overactive bladder: a better understanding of pathophysiology, diagnosis and management. *J Urol* 175: S5-S10.

Yarker YE, Goa KL, and Fitton A (1995) Oxybutynin. A review of its pharmacodynamic and pharmacokinetic properties, and its therapeutic use in detrusor instability. *Drugs Aging* 6: 243-262.

JPET #214031

Footnotes

a)

b)

c) Morihiro Fujimura, Pharmaceutical Research Laboratories, Toray Industries Inc.,

6-10-1 Tebiro, Kamakura, Kanagawa 248-8555 Japan.

E-mail: Morihiro_Fujimura@nts.toray.co.jp

d)

JPET #214031

Figure Legends

Fig. 1. Chemical structure of TRK-130.

Fig. 2. Inhibitory effects of TRK-130, DAMGO, DPDPE, U-69593, morphine, and buprenorphine on forskolin-induced intracellular cAMP accumulation in cells stably expressing human MOR, DOR, or KOR. The values of intracellular cAMP accumulation represent the percentage of change from those recorded after stimulation with 50 μ M of forskolin only. Each plot represents the mean \pm SEM of 6 wells obtained from 3 independent experiments.

Fig. 3. Typical tracings of bladder distension-induced RBCs showing the effects of intravenous TRK-130 (A) or oxybutynin (B) in anesthetized guinea pigs.

Fig. 4. Effects of oral administration of TRK-130 in freely-moving guinea pigs with chemically-induced pollakiuria. (A) Frequency of micturition and mean voided volume per micturition in 3-hour period immediately following TRK-130 administration. Open columns represent pre-drug control values of the respective parameters obtained during the pre-drug session, and filled columns represent post-drug values. (B) Percentage of changes in the frequency of micturition and mean voided volume per micturition obtained during drug session to those obtained during pre-drug control session. Each column represents mean \pm SEM of the results from 8 to 12 guinea pigs per group. †, $P < 0.05$ vs. intravesical injection of saline + vehicle control by Aspin-Welch test. *, $P < 0.025$ vs. vehicle control by one-tailed Williams' test.

JPET #214031

Fig. 5. Effects of subcutaneous administration of TRK-130, morphine, and buprenorphine on the gastrointestinal transit in mice. Each column represents mean \pm SEM of the results from 10 mice per group. *, $P < 0.05$ vs. corresponding vehicle control by two-tailed Shirley-Williams test. †, $P < 0.05$ vs. corresponding vehicle control by two-tailed Williams' test.

JPET #214031

Tables

TABLE 1

Binding affinities of TRK-130 and morphine for human MOR, DOR, and KOR.

K_i values are expressed as means ± SEM of those obtained from 3 experiments.

Compound	<i>K_i</i> value (nM)			Selectivity ratio	
	MOR	DOR	KOR	DOR/MOR	KOR/MOR
TRK-130	0.268 ± 0.012	121 ± 6	8.97 ± 1.05	451	33.5
Morphine	11.5 ± 0.4	1517 ± 150	445 ± 11	132	38.7

JPET #214031

TABLE 2

Agonistic activities of TRK-130, DAMGO, DPDPE, U-69593, morphine, and buprenorphine on forskolin-stimulated cAMP accumulation in human MOR, DOR, and KOR-expressed cells.

EC₅₀ values are defined as the concentration of a compound required for the half maximum inhibition and expressed as means with 95% confidence intervals in parentheses. E_{max} values are expressed as means ± SEM of 6 wells obtained from 3 independent experiments.

Compound	MOR		DOR		KOR	
	EC ₅₀ (nM)	E _{max} (%)	EC ₅₀ (nM)	E _{max} (%)	EC ₅₀ (nM)	E _{max} (%)
TRK-130	2.39 (1.85-3.09)	66.1 ± 3.9 ^{a, b, c}	26.1 (22.4-30.5)	71.0 ± 1.9 ^{d, e}	9.51 (8.40-10.8)	62.6 ± 1.3 ^{d, e}
Morphine	19.9 (18.4-21.5)	100.0 ± 1.0	N.D.	N.D.	N.D.	N.D.
Buprenorphine	4.49 (4.11-4.91)	90.8 ± 1.4 ^{a, b}	8.00 (6.59-9.68)	62.2 ± 2.4 ^d	2.08 (1.53-2.68)	20.5 ± 0.7 ^d
DAMGO	2.07 (1.92-2.23)	100.0 ± 0.9				
DPDPE			0.527 (0.456-0.608)	100.0 ± 0.7		
U-69593					1.64 (1.55-1.73)	100.0±0.4

a, *P*<0.05 vs. the corresponding full agonist; *b*, *P*<0.05 vs. morphine; *c*, *P*<0.05 vs. buprenorphine by Steel-Dwass test. *d*, *P*<0.05 vs. the corresponding full agonist; *e*, *P*<0.05 vs. buprenorphine by Tukey test. N.D., not determined.

JPET #214031

TABLE 3

Effects of TRK-130, morphine, and oxybutynin on the shutdown time of distension-induced RBCs in anesthetized guinea pigs.

ED₃₀ values are expressed as means with 95% confidence intervals in parentheses, and other values as means ± SEM. %MPE (% of maximum possible effect) = {(shutdown time in the drug-treated group - shutdown time in the vehicle-treated group) / [cut-off time (60 minutes) - shutdown time in the vehicle-treated group]} x 100). In 3 and 8 animals treated with TRK-130 and morphine, respectively, the cut-off time value of 60 minutes was used as their shutdown time despite that the disappearance of RBCs after the drug treatment lasted for longer than 60 minutes (cut-off time) in these animals.

Compound	Dose (mg/kg)	n	Shutdown time of bladder contractions (min)		%MPE	ED ₃₀ (mg/kg)
			Predrug	Postdrug		
			Vehicle	6		
TRK-130	0.00125	6	3.3 ± 0.4	4.7 ± 0.9	1.4 ± 1.6	
	0.0025	6	3.2 ± 0.5	18.0 ± 8.5*	25.2 ± 15.2	0.0034
	0.005	6	3.1 ± 0.4	28.0 ± 7.8*	43.0 ± 13.9	(0.0014-0.0048)
	0.01	6	3.3 ± 0.2	45.9 ± 5.5*	74.8 ± 9.8	
Vehicle		11	3.3 ± 0.2	3.5 ± 0.3	0.0 ± 0.5	
Morphine	0.25	10	3.3 ± 0.2	5.5 ± 0.7*	3.5 ± 1.2	
	0.5	10	3.4 ± 0.2	12.4 ± 5.3*	15.7 ± 9.5	0.62
	1	10	3.4 ± 0.2	44.1 ± 8.1*	71.9 ± 14.3	(0.35-0.77)
Vehicle		6	2.8 ± 0.2	4.3 ± 0.7	0.0 ± 1.3	
Oxybutynin	1	6	2.8 ± 0.5	5.0 ± 1.5	1.2 ± 2.7	N.D.

*, *P* < 0.025 vs. corresponding vehicle control by one-tailed Shirley-Williams' test. N.D., not determined.

JPET #214031

TABLE 4

Effects of TRK-130, morphine, and oxybutynin on the intravesical pressure of distension-induced RBCs in anesthetized guinea pigs.

Values are expressed as means \pm SEM. Three and 8 animals treated with TRK-130 and morphine, respectively, had disappearance of bladder contractions for more than 60 minutes (cut-off time); therefore, these animals were excluded from the analysis.

Compound	Dose (mg/kg)	n	Maximum intravesical pressure		% of predrug value
			(cmH ₂ O)		
			Predrug	Postdrug	
Vehicle		6	18.4 \pm 1.3	18.3 \pm 1.1	99.7 \pm 2.2
TRK-130	0.00125	6	18.8 \pm 1.7	18.7 \pm 1.8	99.9 \pm 4.0
	0.0025	5	25.0 \pm 3.6	24.3 \pm 3.1	98.3 \pm 2.1
	0.005	5	22.0 \pm 1.5	22.6 \pm 2.5	102.2 \pm 7.7
	0.01	5	24.2 \pm 1.5	26.9 \pm 3.3	109.6 \pm 7.9
Vehicle		11	24.7 \pm 1.4	23.6 \pm 1.4	95.7 \pm 2.7
Morphine	0.25	10	27.2 \pm 2.8	27.0 \pm 2.9	99.0 \pm 1.6
	0.5	9	25.5 \pm 1.2	25.8 \pm 1.3	101.1 \pm 1.7
	1	3	24.1 \pm 4.5	23.6 \pm 3.7	99.5 \pm 6.3
Vehicle		6	18.7 \pm 2.0	19.4 \pm 2.0	103.9 \pm 1.6
Oxybutynin	1	6	22.7 \pm 1.6	16.6 \pm 1.6	72.7 \pm 3.7*

*, $P < 0.05$ vs. corresponding vehicle control by two-tailed Aspin-Welch test.

JPET #214031

TABLE 5

ID₅₀ values of TRK-130, morphine, and buprenorphine for gastrointestinal transit in mice.

Compound	ID ₅₀ (mg/kg)	95% Confidence interval
TRK-130	N.D.	N.D.
Morphine	1.01	0.695-1.39
Buprenorphine	0.326	0.120-4.17

N.D., not determined.

JPET #214031

Fig. 1

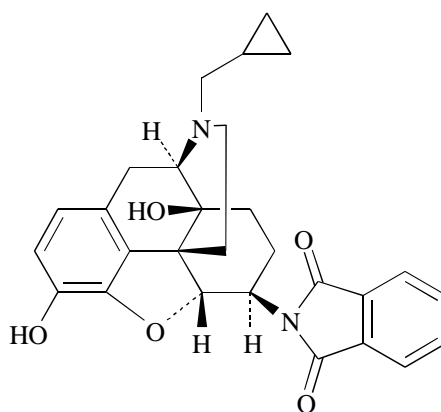


Fig. 2

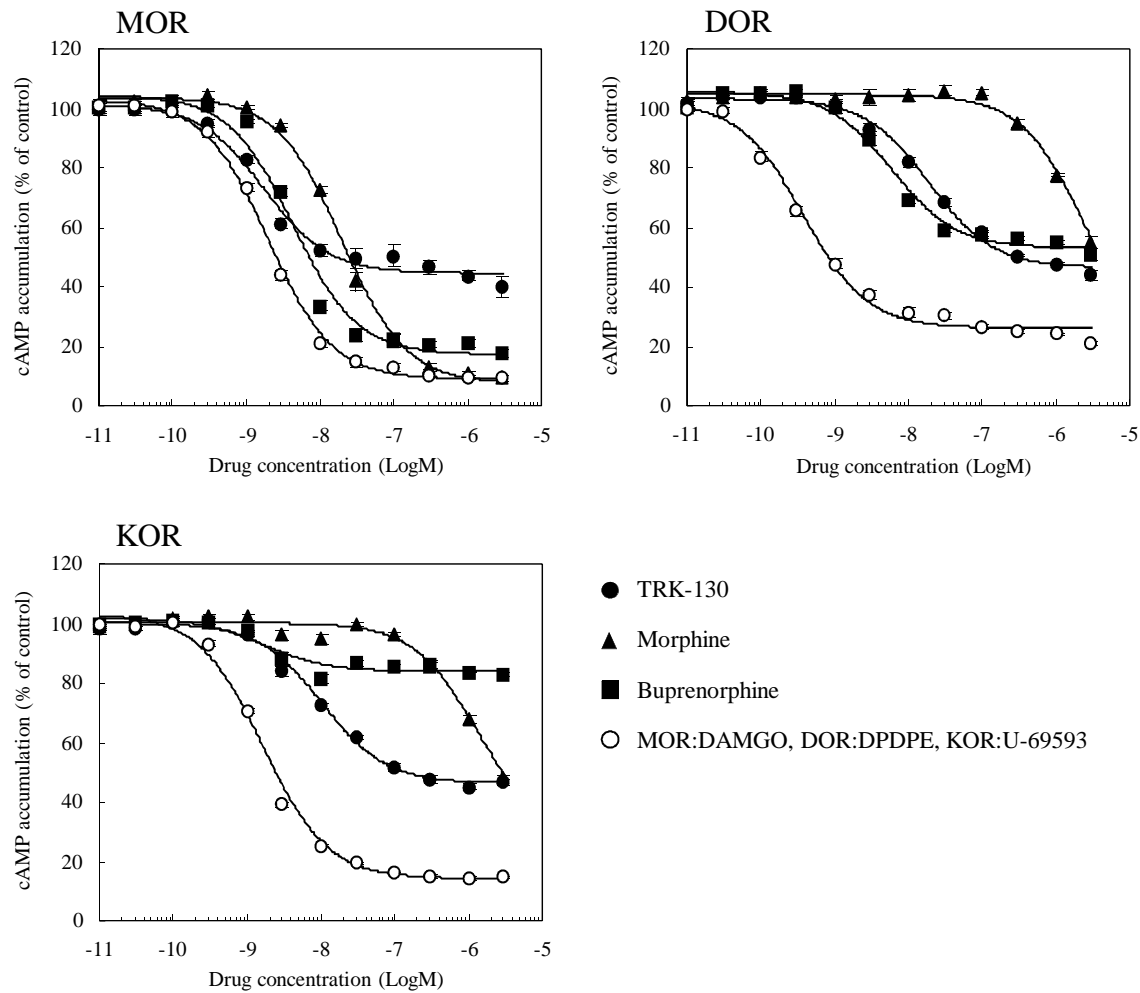


Fig. 3

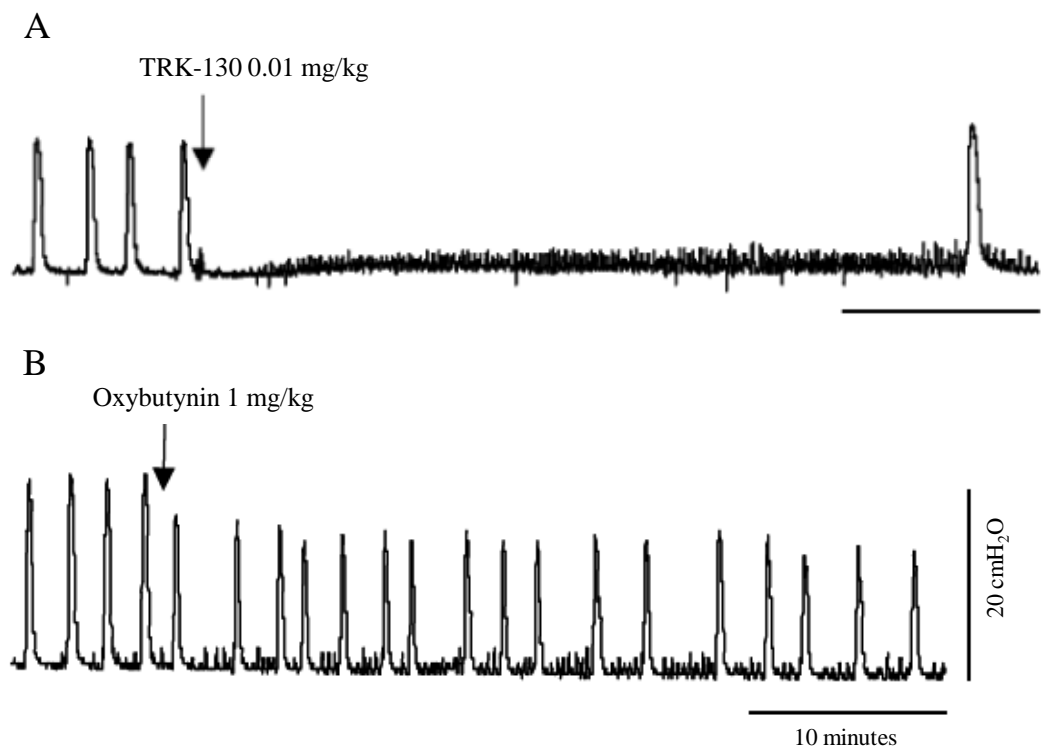


Fig. 4

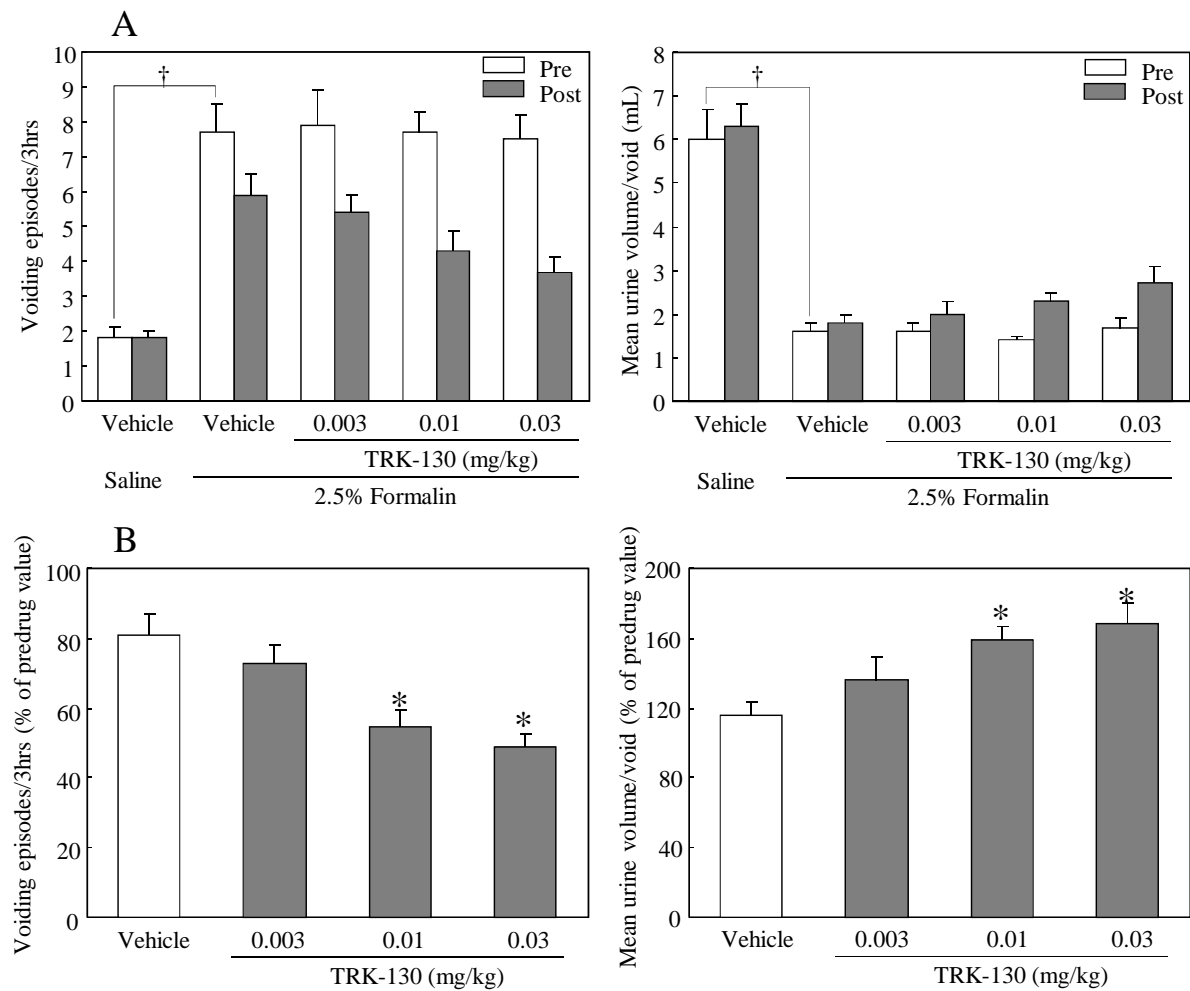


Fig. 5

