The Anti-overactive Bladder Activity of KW-7158 Is Mediated by Blocking Equilibrative Nucleoside Transporter-1

Yoichi Nishiyaa, Tsuyoshi Yamagatab, Ayumu Fukudac, Sachiko Yokokawaa, Takashi Seishic, Takashi Sakumac, Setsuya Sashod, Yukiko Shimizu, Hidetaka Sato, Susumu Sekinea, Masayo Kamigakia, Tetsuo Yoshidaa and Kenji Shibatab,§

a Biologics Research Laboratories, Kyowa Hakko Kirin Co., Ltd.; 3–6–6 Asahi-machi, Machida, Tokyo 194–8533, Japan; b Pharmacological Research Laboratories, Kyowa Hakko Kirin Co., Ltd.; and c Medicinal Chemistry Research Laboratories, Kyowa Hakko Kirin Co., Ltd.; Sunto-gun, Shizuoka 411–8731, Japan.

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KW-7158 is a novel therapeutic candidate for treating overactive bladder (OAB) with a unique mode of action: suppression of sensory afferent nerves. However, the molecular target of this compound remains unknown. We herein report the identification of the KW-7158 target to be equilibrative nucleoside transporter-1 (ENT1). A membrane protein expression library of ca. 7000 genes was expressed in a dorsal root ganglion cell line, which we had previously generated, and subjected to screening for binding with a fluorescent derivative that retains high binding activity to the target. The screening revealed that only cells transfected with an ENT1 expression vector exhibited significant binding. We next performed [3H]KW-7158 binding experiments and an adenosine influx assay and found that KW-7158 binds to and inhibits ENT1. To further demonstrate the pharmacological relevance, we evaluated other known ENT1 inhibitors (nitrobenzylthioinosine, dipyridamole, draflazine) in an in vitro bladder strip contraction assay and the rat spinal cord injury OAB model. We found that all of the inhibitors exhibited anti-OAB activities, of which the potencies were comparable to that of adenosine influx inhibition in vitro. These studies demonstrated that the pharmacological target of KW-7158 is ENT1, at least in the rat OAB model. Our results will aid understanding of the precise mechanism of action of this drug and may also shed new light on the use of the adenosine pathway for the treatment of OAB.

Key words nociceptive neuron; overactive bladder; adenosine transporter; drug target identification

KW-7158 is a drug candidate for overactive bladder (OAB).1–3 Pharmacological studies using rats with xylene-irritated bladders,4 cultured dorsal root ganglion (DRG) neurons,5 and isolated bladder strips6 suggest that KW-7158 has a unique mechanism of action, namely, it inhibits the contraction of the bladder smooth muscle by suppressing the activity of sensory nerves. This unique mode of action distinguishes KW-7158 as a drug candidate that may be free from the side effects caused by other anti-OAB drugs. However, the molecular target of KW-7158 remains elusive.

We have previously shown that the target exists in the rat DRG using [3H]KW-7158 radioligand binding assays.6) To facilitate target identification, we have generated various DRG neuron-like cell lines, including TRD-10 cells, which express a large amount of the target, and TRD-49 cells, which express a negligible amount of the target.6)

In this study, using the DRG cell lines and a KW-7158 fluorescent derivative, we first screened the membrane protein expression library to identify clones expressing KW-7158 binding molecules. This resulted in the identification of a single clone that codes equilibrative nucleoside transporter-1 (ENT1). Further in vitro and in vivo pharmacological studies demonstrated that KW-7158 is a novel ENT1 inhibitor and that the inhibition of ENT1 counteracts overactive bladder in the rat spinal cord injury OAB model.

MATERIALS AND METHODS

Reagents KW-7158 and draflazine were synthesized in our laboratories. Nitrobenzylthioinosine (NBTI), dipyridamole and dilazep were dissolved in dimethyl sulfoxide (DMSO). Atropine and guanethidine (Sigma) were dissolved in distilled water, and capsaicin (Sigma) was dissolved in ethanol. [3H]KW-7158 (703 GBq/mmol) and [3H]adenosine were obtained from GE Healthcare Bioscience.

For the in vivo studies, KW-7158 was treated with an ultrasonicator in 0.5 w/v% methylcellulose 400cP (MC; Wako Pure Chemical Industries, Ltd.). The suspension was administered orally at a volume of 1 mL/kg. Draflazine, dipyridamole and dilazep were suspended in 0.5 w/v% MC.

Animals For the in vitro study, male Crl:CD(SD) rats nine weeks of age were purchased from Charles River Japan and used when they were 10 to 12 weeks old after being acclimated for more than one week. For the in vivo study, female Crl:CD(SD) rats (Charles River Japan) seven weeks of age were purchased and used when they were eight to nine weeks old after being acclimated for one week. All animals were kept at 19–25°C on a 12-h light/dark cycle. Food and water were available ad libitum to all animals. This study was conducted in accordance with the Standards for Proper Conduct of Animal Experiments of Kyowa Hakko Kirin Co., Ltd.

Cell Culture TRD-10/13 cells were recloned by limiting dilution from TRD-10 cells6) for the stable and maximal expression of the target molecule, as determined by a radio-

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lignand binding assay. The cells were cultured as described in our previous report.6

**cDNA Screening** Each clone of the cDNA expression library7 was separately transfected into TRD-49 cells with Lipofectamine 2000 (Invitrogen). After two days, the cells were washed, and binding buffer containing 1 μM of K-025-Cy3 and 0.0028% of digitonin was added. After being incubated for two hours, the cells were washed and subjected to visual inspection with a fluorescent microscope for probe-binding clones.

**Transfection of the ENTI-Expressing Vector** Plasmids harboring the human ENTI gene (FL clone# BNGH42001247) and the human ENT2 gene (FL clone# SKMNC2000966) were obtained from the project described by Ota et al.7 Transfection was performed with Lipofectamine 2000. Cells were cultured until confluency in 6-well plates (binding experiment) or 24-well plates (adenosine uptake measurement). The amount of plasmid was 4 μg/well (3 μg/well for 293T) and 0.8 μg/well, respectively, which was cultured for two days before the experiments. The binding experiments were performed as described in our previous report.8 The values are represented as the mean±S.D.

**ENTI Knockdown Experiment with Small Interfering RNAs (siRNAs)** siRNAs against ENTI were obtained from Qiagen (U.S.A.). The sense sequence of siRNA#1 against rENT1 was r(GAG GCC AUC UGA UAA AUU AdTdT, antisense was r(UAA AUU UAC AGA UGG CUU C) dTdG. For rENT1, the sense sequence was r(UAU UUA AUC AGA UGG CCU C) dTdG, antisense was r(UAG CGG UAG AAC AUC C)dAdA. Control siRNA was obtained from Dharmacon (U.S.A.; siCONTROL Non-targeting). The sequence is from the luciferase gene and the sense strand was r(UUG UCU ACC GAC A) dTdT, antisense was r(UGU CGG UAG UAU GAA GAG AUA CUU). For the binding experiment, TRD-10/13 cells (3×10^4 cells/well) were plated on 6-well plates. The next day, 1 mL of the medium containing 5 μL of Lipofectamine 2000 and 15 μM of siRNA was added to each well. For adenosine uptake measurement, TRD-10/13 cells (6×10^4 cells/well) were plated on 24-well plates. The next day, 0.5 mL of the medium containing 2.5 μL of Lipofectamine 2000 and 15 μM of siRNA was added to each well. The cells were cultured for three days before the experiments.

**Adenosine Uptake** Adenosine uptake was measured based on the method of Leung et al.9 All experiments were carried out in Na+-free buffer containing: 140 mM of N-methyl-β-glucamine, 5 mM of N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES), 5 mM of KH₂PO₄, 1 mM of CaCl₂, 1 mM of MgCl₂ and 10 mM of d-glucose (pH 7.4). Confluent cells in 24-well plates were washed twice in Na+-free buffered solution. Three hundred microliters of Na+-free buffered solution containing 100 nm of [³H]adenosine was then added to each well for one minute. Drugs were added to the cells simultaneously with [³H]adenosine. The plates were then rapidly washed three times with ice-cold phosphate buffered saline (PBS). The cells were solubilized in 0.5 mL of 5% (v/v) Triton X-100. The radioactivity was measured using a liquid scintillation counter. The values are represented as the mean±S.D.

**Electrical Field Stimulation (EFS)-Induced Contraction of Rat Bladder Strips** The in vitro experiment was performed as described in our previous article.9 Briefly, after rat bladder strips were equilibrated in Krebs–Henseleit solution, 1 μM of atropine and 3 μM of guanethidine were added to the organ bath. Then, the stimulation cycles were performed four times as follows: three times at 35-min intervals and once for 40 min after the end of the third stimulation. The ENTI inhibitor or the vehicle DMSO was added to the organ bath five minutes after the second stimulation. Capsaicin (10 μM) was added to the organ bath five minutes after the third EFS to desensitize C-fibers. Fifteen minutes after capsaicin treatment, capsaicin and the tested substances were rinsed away, and atropine and guanethidine were again applied to the organ bath. The fourth EFS was then performed. In the text, the % inhibition values of the effect produced by each compound was calculated with regard to the control responses obtained before the addition of the compound (zero inhibition) and after the addition of capsaicin (100% inhibition). The values are represented as the mean±S.E.M.

**Surgery in Spinal Cord Injury (SCI) Rats** The spinal cord was injured according to the method of Cheng et al.8 Rats were anesthetized with diethyl ether, and the skin on the dorsal surface of the thoracic spinal cord was incised. The 7–8 thoracic vertebrae were laminectomised, and resection was performed approximately 5 mm in length around the 7–8 thoracic spinal cords. The incised area was sutured using surgical silk sutures, and the animals were intramuscularly administered the antibiotic ampicillin (150 mg/kg; Sigma). After surgery for SCI, the urine was manually expressed twice a day and ampicillin was intramuscularly injected once a day until the occurrence of complete self-micturition (for three to four weeks).

**Intravesical Catheter Implantation** The rats were anesthetized with sodium pentobarbital at least three weeks after surgery for SCI. The bladder was exposed using an abdominal midline incision. A catheter filled with physiological saline was inserted into the bladder via the apex and fixed with silk sutures. The other end of the catheter was tunneled subcutaneously, drawn out from the dorsal neck, and fixed to the skin with silk sutures. The rats were subjected to a cystometry study five to seven days after implantation of the intravesical catheter.

**Cystometry** Each animal was housed in a Bollman cage, and a three-way stopcock was connected to the intravesical catheter. One end of the three-way stopcock was connected to a pressure transducer and the other end was connected to a 50-mL syringe set on an infusion pump for infusion of physiological saline. The intravesical pressure was measured with a polygraph system via a strain pressure amplifier and recorded on a thermal array recorder. Sixty to 90 min after completing the preparation for measurement, physiological saline was continuously infused into the urinary bladder for 30 min at a rate of 10 mL/h to confirm the occurrence of micturition contractions. Thirty minutes after cessation of the infusion, physiological saline was infused again to measure the intravesical pressure. Thirty minutes later, the infusion was terminated and the ENTI inhibitor or vehicle was orally administered. Physiological saline was continuously infused for 30 min from 45 min, 165 min and 285 min after the administration. The frequency and amplitude of premicturition contractions and the amplitude of micturition contractions were measured as parameters of intravesical pressure. During a 30-min measurement period, the average maximum increase in intravesical pressure and the number of premicturition contractions per
two minutes corresponding to each voiding contraction were calculated as the values of amplitude and frequency, respectively, of premicturition contractions at each time point. The values of each parameter before administration were taken as 100, and the values obtained at each time point were expressed as percentages of the values obtained before dosing (means±S.E.M.). The statistical analysis was performed and differences were considered to be statistically significant at \( p<0.05 \). A nested analysis of variance was employed to evaluate differences in each contractile parameter between the vehicle group and each test substance group. After the \( F \)-test was performed, Student’s \( t \)-test or the Aspin-Welch test was used.

RESULTS

Generation of a Fluorescent High-Affinity Chemical Probe To identify the target of KW-7158, we initiated a study by conducting cDNA library expression cloning. Since the previous study demonstrated that the pharmacological target of KW-7158 is expressed in the membranes of the DRG and bladder,\(^6\) genes predicted to code membrane proteins \textit{in silico} were chosen from a full-length cDNA expression library.\(^7\) To obtain active chemical probe(s) with which to screen the library, we synthesized K-025, a derivative of KW-7158 that incorporates a linker with a primary amine at the terminal. Figure 1A shows the structure of KW-7158, K-025 and its fluorescent derivative K-025-Cy3. This probe specifically blocked the tonic phase dose-dependently \((i.e., \text{the c-fiber dependent phase})\) at concentrations comparable to KW-7158 in the EFS-induced bladder contraction assay (Fig. 1B). However, conjugating Cy3 to K-025 significantly compromised the binding activity toward TRD-10/13 (data not shown). We reasoned that this is because Cy3 attachment inhibits the probe from penetrating the cell membrane; therefore, we added a very low concentration of digitonin (0.0028%) to the binding reaction.\(^10\) As shown in Fig. 1C, this uncovered the binding of K-025-Cy3 and hence we used this assay condition for cDNA library screening. Altogether, these results suggest that K-025 has the same pharmacological spec-

![Fig. 1. Generation of a Chemical Probe for Target Identification, K-025-Cy3, and the Identification of ENT1 in the cDNA Screening Using the Probe](A) The chemical structures of KW7158, K-025 and its fluorescent derivative K-025-Cy3. (B) Effects of K-025 on EFS-induced bladder contractions \((n=4; \text{**} p<0.05, \text{***} p<0.01 \text{ by Student’s } t\text{-test})\). (C) Inhibition of \([\text{H}]KW-7158\) binding on digitonin-permeabilized TRD-10/13 by K-025-Cy3. (D) Detection of K-025-Cy3 binding in the ENT1-transfected cells using fluorescent microscopy during the membrane protein library screening. Positive cells are indicated by white arrowheads. Scale bar=50 \(\mu\text{m}\).
Membrane Protein Library Screening with the Fluorescent Probe  For the cDNA library screening, the rat immortalized DRG cell line TRD-49, which we established in the previous study as a cell line expressing no KW-7158 pharmacological targets, was used as the host cell. Using the microwell format, in which each cDNA clone was transfected in TRD-49 cells individually, we screened approximately 7000 genes and examined K-025-Cy3 binding by visual inspection under a fluorescent microscope. The only clone we found to bind to the probe was equilibrative nucleoside transporter-1 (ENT1; Fig. 1D). ENT1 has 456 amino acid residues with 11 predicted transmembrane domains and is a member of the equilibrative transporter family that mediates cellular uptake of nucleosides from the surrounding medium.

Radioligand Binding Assays Showed that KW-7158 Binds to ENT1 To prove that the observed binding was not an artifact caused by the derivatization, we expressed the human ENT1 gene on 293T and TRD-49 cells and measured [3H]KW-7158 binding. As shown in Fig. 2A, the introduction of the human ENT1 (hENT1) gene expression vector, but not the control vector, increased specific binding by 2-fold and 4-fold for 293T and TRD-49, respectively. To confirm that the binding observed in TRD-10/13 (a DRG cell line that expresses a high amount of KW-7158 binding molecules) was derived from ENT1, we examined the effects of ENT knockdown by siRNAs on the specific binding of [3H]KW-7158. As shown in Fig. 2B, the specific binding decreased by 40% following the introduction of two different siRNAs (the mRNA level and amount of adenosine uptake were decreased by 60% and 40%, respectively (Supplementary Figs. S1A, B). Therefore, the specific binding observed in TRD-10/13 was, at least

Fig. 2. Validation of ENT1 as the Target Molecule Using Radioligand Binding Experiments and Adenosine Influx Assays

(A) Specific binding of 10nM [3H]KW-7158 on 293T or TRD-49 cells transfected with the human ENT1 gene. (B) Effects of siRNAs on 10nM [3H]KW-7158 binding in TRD-10/13 cells. (C) Concentration-dependent inhibition of adenosine uptake by KW-7158. (D) Binding studies of 10nM [3H]KW-7158 on human ENT1- or ENT2-expressed TRD-49 in the presence (nonspecific) or absence (total) of 10µM of KW-7158. (E) Effects of 1µM of NBTI on the concentration-dependent inhibition of the adenosine uptake by KW-7158 using TRD-10/13 cells. All experiments were conducted in duplicate or triplicate.
in part, due to binding to ENT1.

KW-7158 Inhibited the Adenosine Uptake through ENT1 To test whether KW-7158 modulates adenosine transport through ENT1, we first established an adenosine incorporation assay using TRD-10/13. There are two pathways through which adenosine is incorporated into cells, the sodium-independent ENT pathway and the sodium-dependent concentrative nucleoside transporter pathway. It is known that at 100 nM, NBTI inhibits ENT1 only, whereas at 500 µM, it inhibits both ENT1 and ENT2. Therefore, we conducted the assay at these concentrations and found that half of the incorporation of adenosine by TRD-10/13 was inhibited by 100 nM of NBTI, while all of the incorporation was inhibited by 500 µM of NBTI, suggesting that the incorporation was mediated equally through ENT1 and ENT2 (data not shown).

Using this system, KW-7158 inhibited adenosine uptake dose-dependently from 5 nM, and at 50 µM, it inhibited 80% of the uptake (Fig. 2C). Therefore, it was shown that KW-7158 inhibits adenosine uptake through ENTs.

We next examined the selectivity of KW-7158 between ENT1 and ENT2 using NBTI. We introduced hENT1 or hENT2 to TRD-49 cells and performed binding experiments...
with 10 nM [3H]KW-7158. Although both genes were expressed at least at the mRNA level (data not shown), we observed significant specific binding only when hENT1 was transfected (Fig. 2D). Then, we tested the effects of KW-7158 on adenosine uptake in the presence of 1 µM of NBTI, at which concentration it can be extrapolated that the uptake is only through ENT2. As shown in Fig. 2E, the uptake was inhibited by high concentrations of KW-7158 only (> 500 nM). Therefore, it is probable that the inhibition elicited by low concentrations of KW-7158 (< 500 nM) in the absence of NBTI occurs through ENT1.

The Potency of Inhibition of Adenosine Uptake Was Correlated with That of the EFS-Induced Bladder Contraction Model If inhibition of adenosine uptake through ENT1 leads to inhibition of EFS-induced bladder contraction, other ENT1 antagonists should also be able to elicit these activities. Indeed, all of the ENT1 inhibitors we tested (NBTI, drafazine, dipyridamole, dilazep) exhibited significant activities in EFS contraction (Fig. 3A). Furthermore, the potencies observed in the EFS-induced bladder contraction model were parallel to those seen in the in vitro adenosine uptake experiments (Figs. 3A, B). These results strongly suggest that the effects of KW-7158 on EFS-induced bladder contraction are mediated by the inhibition of ENT1.

KW-7158 and Other ENT1 Inhibitors Were Effective in the Rat SCI OAB Model The above findings were further validated in the rat spinal cord injury model of OAB. In the SCI rats, 0.1 mg/kg of KW-7158 suppressed the premicturition frequency/amplitude (Fig. 4A) without affecting the amplitude of voiding contractions (data not shown). These effects appeared as early as one hour postadministration and persisted until at least five hours. Using the same model rats, we observed that two other ENT1 inhibitors, dipyridamole and dilazep, both exhibited the same inhibitory activities, albeit at higher doses of 300 mg/kg (Fig. 4B). NBTI and other ENT1 inhibitors also had similar effects in both OAB models (data not shown). These results demonstrate that the anti-OAB effect of KW-7158 is, at least in part, mediated through ENT1.

DISCUSSION

In pathological states such as spinal-cord injury or bladder hyperplasia, the extension stimuli of the bladder wall are transmitted afferently through c-fiber, then parasympathetic nerves efferently contract the bladder detrusor muscle. Pharmacological studies of rats with xylene-irritated bladders and isolated bladder strips have suggested that the effects of KW-7158 are mediated through the inhibition of sensory c-fiber. Therefore, inhibition of adenosine transport by KW-7158 should somehow inhibit the neuronal activity of c-fiber.

The direct effect of inhibiting ENT1 is to increase the concentration of extracellular adenosine. It is well known that adenosine modulates neuronal activity and synaptic transmission through adenosine receptors (A1, A2a, A2b, A3). With regard to overactive bladder, an A2a antagonist acts at supraspinal sites and increases the intercontraction interval in a rat model of Parkinson’s disease. Moreover, there are several reports suggesting the extracellular adenosine modulation by ENT1 and its effect on central neuronal activities. On the other hand, as for the peripheral nervous system, although ENT1 is reported to be expressed in the cell bodies of peptidergic neurons in the DRG, there are few reports studying the effects of ENT1 inhibitors on peripheral neurons. Therefore, the physiological role of ENT1 in peripheral neurons, especially afferent sensory nerves, remains to be elucidated.

As for the possible effects of adenosine itself on peripheral nerves, it has been reported in rodents that activation of the A1 receptor has an analgesic effect, while activation of the A2 receptor promotes pain. It has also been suggested that adenosine and A1 agonists exert sedative effects on ß- and c-fiber and that adenosine exerts analgesic effects in peripheral tissues. Therefore, it is probable, at least under some pathological or physiological conditions, that increases in the adenosine concentration induced by inhibiting ENT1 can lead to the inhibition of peripheral afferent c-fiber.

Adenosine is produced in various tissues and organs and is involved in energy demand supply and the modulation of physiological reactions. In addition, ENT1 is expressed not only in the DRG, but also in various tissues such as the heart, mammary glands, placenta and liver as well as the nervous system. Therefore, it is likely that various responses as well as inhibition of OAB are evoked if the concentration of extracellular adenosine is increased by the inhibition of ENT1. In fact, an ENT1 inhibitor, dipyridamole, exhibits coronary dilating effects and antiplatelet aggregation activity, while drafazine exhibits myocardial protection effects during ischemia in humans. Whether or not KW-7158 also exerts these pharmacological effects awaits further investigation.

According to a report, KW-7158 enhances the A-type K current of primary DRG neurons. It is true that increases in the A-type K current inhibit depolarization of neurons, which is considered to explain the inhibitory activity of c-fiber induced by KW-7158. In addition, according to a study by Liu et al., the 4-AP-sensitive Kv channel (hence pharmacologically classified as the A-type K channel) opens with adenosine, while the opening time is prolonged by NBTI. Therefore, the A-type current potentiation caused by KW-7158, as reported in the reference, may be mediated through EN T1 blockade.

Our studies demonstrated that the pharmacological target of KW-7158 is ENT1, at least in the rat OAB model. KW-7158 inhibits adenosine uptake through ENT1 expressed in peripheral c-fiber, thereby possibly causing a local increase in extracellular adenosine. This rise may activate subtypes(s) of adenosine receptors and/or may open A-type K channels, resulting in the inactivation of c-fiber and eventually inhibiting OAB.

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REFERENCES

1) Yamagata T, Atsuki K, Ohno T, Shirakura S, de Groat WC, Sculpond A, Karasawa A, Yoshimura N. Agent for the treatment of overactive bladder. International Patent Application WO02/078523 (2002).
2) Yamagata T, Atsuki K, Ohno T, Shirakura S, de Groat WC, Yo-
9) Cheng CL, Ma CP, de Groat WC. Effect of capsaicin on micturition in rats. *Brain Res.*, **946**, 72–78 (1998).

10) Stearns ME, Ochs RL. A functional *in vitro* model for studies of vesicular exocytosis. *J. Cell Biol.*, **94**, 727–739 (1982).

11) Griffiths M, Beaumont N, Yao SY, Sundaram M, Boumeh CE, Davies A, Kwong FY, Coe I, Cass CE, Young JD, Baldwin SA. Cloning of a human nucleoside transporter implicated in the cellular uptake of adenosine and chemotherapeutic drugs. *Nat. Med.*, **3**, 89–93 (1997).

12) Yao SY, Ng AM, Muzyka WR, Griffiths M, Cass CE, Baldwin SA, Young JD. Molecular cloning and functional characterization of nitrobenzothionoisone (NBMPR)-sensitive (es) and NBMPR-insensitive (ei) equilibrative nucleoside transporter proteins (eENT1 and eENT2) from rat tissues. *J. Biol. Chem.*, **272**, 28423–28430 (1997).

13) Kong W, Engel K, Wang J. Mammalian nucleoside transporters. *Curr. Drug Metab.*, **5**, 63–84 (2004).

14) Kato R, Maeda T, Akaike T, Tanai M. Nucleoside transport at the blood-tissue barrier studied with primary-cultured sertoli cells. *J. Pharmacol. Exp. Ther.*, **312**, 601–608 (2004).

15) Yoshimura N. Bladder afferent pathway and spinal cord injury: possible mechanisms inducing hyperreflexia of the urinary bladder. *Prog. Neurobiol.*, **57**, 583–606 (1999).

16) Cunha RA. Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors. *Neurochem. Int.*, **30**, 107–125 (2001).

17) Jacobson KA, Gao ZG. Adenosine receptors as therapeutic targets. *Nat. Rev. Drug Discov.*, **5**, 247–264 (2006).

18) Latini S, Pedata F. Adenosine in the central nervous system: release mechanisms and extracellular concentrations. *J. Neurochem.*, **79**, 453–484 (2001).

19) Ohay ME, Stiles GL. The role of receptor structure in determining adenosine receptor activity. *Pharmacol. Ther.*, **85**, 55–75 (2000).

20) Kitta T, Chancellor MB, de Groat WC, Kuno S, Nonomura K, Yoshimura N. Suppression of bladder overactivity by adenosine A2A receptor antagonist in a rat model of Parkinson’s disease. *J. Urol.*, **187**, 1890–1897 (2012).

21) Ackley MA, Governo RJ, Cass CE, Young JD, Baldwin SA, King AE. Control of glutamatergic neurotransmission in the rat spinal dorsal horn by the nucleoside transporter ENT1. *J. Physiol.*, **548**, 507–517 (2003).

22) Liu Y, Fujita T, Kawasaki Y, Kumamoto E. Regulation by equilibrative nucleoside transporter of adenosine outward currents in adult rat spinal dorsal horn neurons. *Brain Res. Bull.*, **64**, 75–83 (2004).

23) Governo RJ, Deuchars J, Baldwin SA, King AE. Localization of the NBMPR-sensitive equilibrative nucleoside transporter, ENT1, in the rat dorsal root ganglion and lumbar spinal cord. *Brain Res.*, **1059**, 129–138 (2005).

24) Karlsten R, Gohd T, Post C. Local antinociceptive and hyperalgesic effects in the formalin test after peripheral administration of adenosine analogues in mice. *Pharmacol. Toxicol.*, **40**, 434–438 (1992).

25) Taiwo YO, Levine JD. Direct cutaneous hyperalgesia induced by adenosine. *Neuroscience*, **38**, 75–78 (1992).

26) Luo J, Kawasaki Y, Yang K, Fujita T, Kumamoto E. Modulation by adenosine of Adelta and C primary-afferent glutamatergic transmission in adult rat substantia gelatinosa neurons. *Neuroscience*, **125**, 221–231 (2004).

27) Goldman N, Chen M, Fujita T, Xu Q, Peng W, Liu W, Jensen TK, Pei Y, Wang F, Han X, Chen JF, Schernhammer J, Takano T, Bekar L, Tseu K, Nedergraaff M. Adenosine A1 receptors mediate local anti-nociceptive effects of acupuncture. *Nat. Neurosci.*, **13**, 883–888 (2010).

28) De Schryver EL, Algra A, van Gin J. Cochrane review: dipyridamole for preventing major vascular events in patients with vascular disease. *Stroke*, **34**, 2072–2080 (2003).

29) Ottervanger JP, Gregor P, Widimsky P, Zijlstra F, Kluiver EP, Hof AW, Suryapranata H. Cardioprotective effects of adenosine transport inhibition during reversible ischaemia in patients with coronary artery disease. *Int. J. Cardiovasc. Intervent.*, **2**, 181–186 (1999).