A Novel Analgesic Compound OT-7100 Attenuates Nociceptive Responses in Animal Models of Inflammatory and Neuropathic Hyperalgesia: A Possible Involvement of Adenosinergic Anti-nociception

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ABSTRACT—We studied the effects of OT-7100 (5-n-butyl-7-(3,4,5-trimethoxybenzoylamino)pyrazolo[1,5-a]pyrimidine), a novel analgesic compound, on the inhibitory action of adenosine on the contraction of guinea pig ileum and investigated the effects of OT-7100 on the nociceptive responses in animal models of inflammatory and peripheral neuropathic hyperalgesia and decreases spinal c-Fos expression. OT-7100 at 0.3–3 μM significantly enhanced the inhibitory effect of adenosine on the contraction of guinea pig ileum. The efficacy of OT-7100 (1, 3 or 10 mg/kg, p.o.) on hyperalgesia induced by yeast or substance P and in the Bennett model was significantly suppressed by coadministration of the adenosine A1 antagonist DPCPX (0.01 or 0.1 pmol/animal, i.t.), while OT-7100 without DPCPX significantly increased the nociceptive threshold in each rat model. OT-7100 (3, 10 and 30 mg/kg per day, p.o.) significantly inhibited the mechanical nociceptive threshold in the injured paw in the Chung model. OT-7100 (30 mg/kg, p.o.) significantly decreased the number of Fos-LI neurons in the spinal dorsal horn in the Bennett model. These findings suggest that OT-7100 inhibits hyperalgesia in these animal models possibly by enhancing adenosinergic neurotransmission in the dorsal horn, although we still lack direct evidence for it.

Keywords: Adenosine, OT-7100, Hyperalgesia, Analgesic

Peripheral neuropathic pain resulting from peripheral nerve injury is a chronic condition that usually cannot be relieved with conventional analgesics such as non-steroidal anti-inflammatory drugs (NSAIDs) (1). Regarding opioids, there are cases where no pain relief was observed and in cases where pain relief was observed, a problem of side effects has been pointed out (2). Patients with peripheral nerve injuries frequently complain of sensory abnormalities, including an unpleasant abnormal sensation (dysesthesia), an increased response to a stimulus that is normally painful (hyperalgesia), and pain due to a stimulus which does not normally provoke pain (allodynia) (1). This syndrome is defined as peripheral neuropathic pain. These symptoms are frequently observed after trauma or surgery and also observed in patients with postherpetic neuralgia, trigeminal neuralgia, or painful diabetic neuropathy. It is known that peripheral neuropathic pain cannot be controlled with conventional analgesic agents, and it is therefore treated with drugs such as antidepressants or anticonvulsants, even though side effects related to the central effects of these drugs are common. Although the mechanism responsible for the development of peripheral neuropathic pain is not yet understood, significant progress with regard to its pathogenesis has been made since the introduction of animal models of this type of pain, including the chronic constriction injury (CCI) model of Bennett and Xie (3) and the selective spinal neurectomy model of Kim and Chung (4). Partial nerve injury is the basic mechanism used for the induction of chronic hyperalgesia in these models. The main pathological feature of the neuropathy seen in these animal models is an axonopathy due to Wallerian degeneration with a loss of myelinated fibers, a subsequent increase in thinly myelinated regenerating fibers, and

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neuroma formation (5–9). Recently, many trials have been carried out to develop effective new drugs for the treatment of peripheral neuropathic pain using these animal models. However, no agent has been found that has a specific effect on neuropathic hyperalgesia without affecting normal nociceptive thresholds.

We previously reported that OT-7100 markedly increased the mechanical nociceptive threshold in the injured paw without affecting the nociceptive threshold in the contralateral uninjured paw in the Bennett model, probably through a mechanism that has yet to be clarified but may be quite different from that of other analgesics (10). In addition, OT-7100 has been found to be effective in acute hyperalgesia induced by yeast and substance P in rats. These results suggested that OT-7100 is a new type of analgesic for the treatment of uncontrollable pain, such as peripheral neuropathic pain. Regarding the mechanism of action, there was little data at the beginning of this study, except for our previous report that OT-7100 has no inhibitory effects on cyclooxygenase up to 100 μM and no binding affinity with the substance P receptor up to 1 mM (10). However, it was recognized that OT-7100 has a pyrazolopyrimidine structure that is considered to have a (10). Therefore, we focused our study on the relation between adenosine and OT-7100. We studied the effects of OT-7100 on the inhibitory effects of adenosine on contraction of guinea pig ileum by electrical stimulation. After confirming the enhancement of adenosine’s effect by OT-7100 in vitro, we then investigated whether the anti-hyperalgesic action of OT-7100 on yeast-, substance P- or CCI-induced hyperalgesia also involves adenosine’s effects or not. In addition, we studied the dose-response of the effect of OT-7100 using the Chung model of peripheral neuropathic pain. Furthermore, the expression of c-Fos protein encoded by the immediate-early gene, c-fos has been widely used as a marker in studying the effects of analgesic compounds (16), and c-Fos-immunoreactive neurons are shown to increase in the spinal cord in the Bennett model (17). Thus, we also investigated the effect of OT-7100 on c-Fos expression in the spinal cord using the Bennett model of peripheral neuropathic pain in the present study.

MATERIALS AND METHODS

Animals
Male Hartley guinea pigs at 6 to 9 weeks of age (Charles River Japan, Inc., Yokohama) were used in the in vitro study. In the in vivo study, male Sprague-Dawley (SD) rats at 5 to 8 weeks of age (Charles River Japan, Inc.) were also used. For the measurement of spinal c-Fos expression, 14 male SD rats at 5 weeks of age (SLC Japan, Shizuoka) were used. The guinea pigs and rats were housed in aluminum or plastic cages under conditions of controlled temperature (23 ± 2°C) and humidity (55 ± 10%) with a 12-h light/12-h dark cycle during the study period. The rats were individually housed in aluminum cages under a 12-h light/dark cycle during the study period. Experiments were performed in accordance with the Declaration of Helsinki.

In vitro study
The guinea pigs were sacrificed by exsanguination under ether anesthesia, and the ileum was immediately excised. A segment of ileum was washed in Krebs-Henseleit solution, and the longitudinal muscle was excised. Platinum electrodes were attached to the muscle segment, which was suspended in a Magnus tube containing Krebs-Henseleit solution at 37°C and aerated with 95% O₂ and 5% CO₂. The muscle segment was electrically stimulated (0.2 Hz) with a stimulator (DPS-06; Dia Medical System Co., Ltd., Tokyo) and the induced contraction was recorded with an FD Pickup (TB-611T; Nihon Koden Kogyo Co., Ltd., Tokyo) on a Unicorder (U-228; Nippon Denshi Kagaku Co., Ltd., Kyoto). When the response had stabilized, adenosine (Wako Pure Chemical Industries, Ltd., Osaka) was cumulatively added to the tube at final concentrations ranging from 0.1 to 100 μM, and a dose-response curve was generated (control specimens). In the OT-7100 specimens, OT-7100 was added to the Magnus tube in advance at final concentrations of 0.3, 1 and 3 μM. Adenosine was cumulatively added to the tube as described above, and dose-response curves were generated. ED₅₀ values were calculated as the concentration of adenosine inhibiting contraction by 50%. Ten repeated experiments were conducted using the same procedure.

In vivo study

Yeast-induced hyperalgesia model: A 20% suspension of Brewer’s yeast (Sigma Chemical Co., St. Louis, MO, USA) suspended in 0.9% saline solution (Otsuka Pharmaceutical Co., Ltd., Tokyo) was injected at 0.1 mL/site into the plantar surface of the left hind paw as described by Randall and Selitto (18).

Substance P-induced hyperalgesia model: Substance P (Sigma Chemical Co.) was dissolved in 0.9% saline solution and administered to the rats at 6 weeks of age by subcutaneous injection into the plantar surface of the left hind paw at 25 ng (0.1 mL)/site.

CCI-induced hyperalgesia model (Bennett model): For preparation of the Bennett model, 5-week-old rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.; Dainippon Pharmaceutical Co., Ltd., Osaka). The left common sciatic nerve was exposed at mid thigh by blunt
Drug administration: OT-7100 was administered as a single oral dose of 3 mg/kg in the yeast-induced hyperalgesia model or 1 mg/kg in the substance P-induced hyperalgesia model. OT-7100 was administered orally at a dose of 10 mg/kg for 3 or 7 consecutive days in the Bennett model. Amitriptyline was administered orally at a dose of 30 mg/kg for 5 consecutive days in the Bennett model. Dipyridamole was administered orally once daily for 7 consecutive days at dosages of 100 and 300 mg/kg. A 5% acacia solution at 10 mL/kg was used as the control substance in each experiment. An adenosine A1 antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; Research Biochemicals International) and an adenosine A2 antagonist, 3,7-dimethyl-1-propargylxanthine (DMPX, Research Biochemicals International) were dissolved in dimethyl sulfoxide (DMSO; Wako Pure Chemical Industries, Ltd.), then diluted with Ringer’s solution (Otsuka Pharmaceutical Co., Ltd.) for intrathecal administration. Since the concentration of DMSO in the DPCPX or DMPX injection solution was adjusted to 1%, the concentration of DMSO in the Ringer’s solution for controls was also adjusted to 1%. DPCPX was administered intrathecally at a dose of 0.1 pmol/animal in the yeast- and substance P-induced hyperalgesia models or 0.01 pmol/animal in the Bennett model in combination with the administration of OT-7100 or amitriptyline. DMPX was administered intrathecally at a dose of 0.1 pmol/animal in the Bennett model in combination with the administration of OT-7100 or amitriptyline. DMPX was administered intrathecally at a dose of 0.1 pmol/animal in the Bennett model in combination with the administration of OT-7100. Ringer’s solution at 100 µL/animal was injected in the controls. Rats that received 5% acacia solution alone (10 mL/kg) served as controls. DPCPX, DMPX or Ringer’s solution was administered intrathecally 1 h before the oral administration of OT-7100, amitriptyline or acacia solution.

Behavioral testing: Mechanical nociceptive thresholds were assessed before and after injection, for 5 h in the yeast-induced hyperalgesia model or 60 min in the substance P-induced hyperalgesia model, using the pressure stimulation method described by Randall and Selitto (18). Seven or 9 animals were assigned to each group in the yeast- or substance P-induced hyperalgesia model experiments, respectively. In the case of the Bennett model, mechanical nociceptive thresholds were assessed from 2 weeks after surgery. The nociceptive threshold in the injured paw was measured before and at 3 h as previously described (10) after dosing on days 1 and 3 or 1, 3, 5 and 7 for OT-7100; on days 1 and 5 for amitriptyline; or on days 1, 3, 5 and 7 for dipyridamole; and the nociceptive threshold in the uninjured paw was measured more than 30 min later. If an increase in nociceptive threshold was observed, the nociceptive threshold was measured daily for 3 or 4 consecutive days after the end of the administration period in order to confirm the absence of a prolonged effect. For pre-dosing values, nociceptive thresholds in both paws in the Bennett model were determined three times before the start of drug administration. Hyperalgesia was observed in the injured paw of all rats that underwent CCI surgery. However, only rats in which the difference in nociceptive threshold between the left (injured) paw and the right (uninjured) paw was stable (i.e., in which the variation in the difference between the injured paw and uninjured paw in three measurements was 11 mmHg or less) and which showed a nociceptive threshold of 30 mmHg or less in the injured paw and 40 mmHg or more in the uninjured paw were used in the study. Four to 7 animals were assigned to each group in the Bennett-model experiment.

Segmental spinal nerve ligation-induced mechanical hyperalgesia

Preparation of the Chung model: The animals were anesthetized with sodium pentobarbital (40 mg/kg, i.p.). They were then placed in the prone position and the left paraspinal muscles were dissected away from the spinous processes in the range from L-4 to S-2. The left transverse process of L-6 was carefully removed with a small rongeur to expose the spinal nerves of L-4 to L-6. The left spinal nerves L-5 and L-6 were isolated and tightly ligated with 5-0 nylon suture.

Behavioral testing: Mechanical hyperalgesia was assessed 2 weeks after surgery. The nociceptive threshold in each paw of individual rats was determined three times using the pressure stimulation method described by Randall and Selitto (18). Hyperalgesia was seen in the injured paw in all operated rats. However, rats in which the difference in nociceptive threshold between the left (injured) paw and the right (uninjured) paw was stable (i.e., in which the variation in the difference between the injured paw and uninjured paw in three measurements was 10 mmHg or less) and which showed a nociceptive threshold of 30 mmHg or less in the injured paw and 40 mmHg or more in the uninjured paw were selected for the experiments (n = 28). There was no significant difference in nociceptive threshold in the uninjured paws between the experimental rats and control rats. Seven rats were assigned to each group.

Drug administration and nociceptive threshold assessment: OT-7100 was administered orally to rats once daily for 14 consecutive days at dosages of 3, 10 and 30 mg/kg per day. Rats that received 5% acacia solution alone (10 mL/kg) served as controls. The nociceptive threshold was measured 3 h after dosing on days 1, 3, 5, 7, 9, 11...
and 14 and also daily during the 5-day recovery period after the final dose.

**Fos immunohistochemistry**

On day 0, all rats underwent operation on the left side following the procedures originally described by Bennett and Xie (3). Briefly, the middle third of the ipsilateral sciatic nerve (left side) was exposed through a 1.5-cm longitudinal skin incision. With the help of an operating microscope, four 0-4 chromic gut ligatures were loosely placed around the sciatic nerve. On the contralateral side, the sciatic nerve was exposed but not injured (sham operation). The wound was then closed with interrupted 3-0 silk sutures. The rats were further divided into three groups at random as follows: In the prophylactic treatment group, rats \( n = 5 \) received OT-7100 (30 mg/kg) orally once daily from day −3 to day +7. OT-7100 (30 mg/kg) was orally administered to rats in the therapeutic group \( n = 4 \) from day +3 to day +7. Rats that received 5% acacia solution alone \( (10 \text{ mL/kg}) \) from day −3 to day +7 served as the control group \( n = 5 \). Systemic oral administration of OT-7100 at a dose of 30 mg/kg significantly normalized the nociceptive threshold in CCI-model rats (10). On day +7, 3 h after drug administration, all animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused transcardially with 200 mL of saline solution followed by 500 mL of 4% paraformaldehyde (PFA) at 4°C in 0.1 M phosphate-buffered saline (PBS) at PH 7.4. The L-5 spinal cord segment of each rat was then excised. These samples were postfixed in the same fixative for 16 h and stored in 30% (w/v) sucrose in 0.1 M PBS for 16 h at 4°C. After fixation, the samples were embedded in O.C.T compound (Sakura Finetehcnical Co., Ltd., Tokyo) and rapidly frozen with powdered dry ice and stored at −80°C. Transverse 30-μm-thick sections were cut using a cryostat at −20°C and collected in cold 0.1 M PBS. The sections were immunostained for Fos protein using the avidin-biotin-peroxidase complex (ABC) method. They were then incubated for 40 h at 4°C with rabbit polyclonal anti-Fos antibody (Ab-5; Oncogene Research Products, Boston, MA, USA) diluted 1:1000 with PBS containing 5% normal goat serum and 0.3% Triton X-100. After rinsing the sections were further incubated for 90 min with biotinylated goat anti-rabbit IgG (1:200) and then with avidin-biotin-peroxidase complex (Vectastain ABC kit; Vector Laboratories, Inc., Burlingame, CA, USA) for 60 min. Peroxidase in the tissue sections was visualized using 0.05% diaminobenzidine containing 0.2% nickel ammonium sulfate. After rinsing in Tris-HCl-buffered saline (TBS), the sections were mounted on glass slides, air dried and coverslipped with mounting medium. Under bright-field illumination, Fos-positive neuronal nuclei were distinguished as homogeneous gray-black elements with a well-defined border. It should be noted that the terms c-fos expression and Fos-LI are used interchangeably and are meant to be synonymous with Fos-positive neuronal nuclei. The number of neurons showing Fos-LI nuclei was counted in five sections randomly selected from the sections at the L-5 level of the spinal cord in each animal.

To quantitate changes in the number of Fos-LI neurons in different laminae of the spinal cord, which were divided into three regions based on functional differences: 1) the superficial dorsal horn, laminae 1 and 2, which contains predominantly nocireceptive cells (19, 20); 2) laminae 3 and 4, which contain cells predominantly responsive to non-noxious mechanical stimuli (20–23); and 3) the deeper laminae 5, 6 and 7 (20), which contain wide-dynamic-range and nociceptive-specific cells (deeper layer). The counts were made by an investigator who was blinded to the specific treatment that the animals had received.

**Drug**

OT-7100 \((5-n\text{-butyl}-7-(3,4,5\text{-trimethoxybenzoylamino})\text{pyrazolo}[1,5-a]pyrimidine; molecular weight 384.44)\) was synthesized at Otsuka Pharmaceutical Factory, Inc. (Tokushima). Amitriptyline (a tricyclic anti-depressant) was purchased from Sigma Chemical Co. Dipyridamole was purchased from Wako Pure Chemical Industries, Ltd. OT-7100, amitriptyline and dipyridamole were suspended in 5% acacia solution.

**Statistical analyses**

In the in vitro study, data are expressed as the mean ± S.D. Bartlett’s test was used to assess the homogeneity of variance in ED\(_{50}\) values. After the data were confirmed not to be homogeneous, the effect of OT-7100 on ED\(_{50}\) values was analyzed by the Friedman test using Statistica statistical software (Stat Soft, Inc., Tulsa, OK, USA). In the in vivo study, data are expressed as the mean ± S.D. Differences between three or more groups were assessed by Dunnett’s multiple comparison test. Values of \( P<0.05 \) were considered statistically significant. In the c-Fos experiment, data are expressed as the mean ± S.D. One-way analysis of variance (ANOVA) with post-hoc tests (Fisher’s PLSD) was used to investigate significant differences, which were considered significant at \( P<0.05 \).

**RESULTS**

**Enhancement of inhibitory effects of adenosine by OT-7100**

OT-7100 at 0.3 – 3 μM significantly enhanced the inhibitory effect of adenosine on the contraction of guinea pig ileum longitudinal muscle induced by electrical stimulation \((P<0.001,\ Table\ 1)\).
Table 1. ED$_{50}$ values of adenosine in inhibiting contraction of guinea pig ileum in the presence of OT-7100

<table>
<thead>
<tr>
<th>Concentration of OT-7100</th>
<th>0</th>
<th>0.3 μM</th>
<th>1 μM</th>
<th>3 μM</th>
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<tr>
<td>ED$_{50}$ (μM)</td>
<td>16.7 ± 21.0</td>
<td>9.9 ± 16.4</td>
<td>4.6 ± 8.4</td>
<td>1.5 ± 2.0</td>
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Data are expressed as the mean ± S.D., n = 10 per group.

Effects of the adenosine A$_1$ antagonist, DPCPX, on anti-hyperalgesic activity of OT-7100

Effects of DPCPX on anti-hyperalgesic effects of OT-7100 in the yeast model: Nociceptive thresholds in the paws in the group that received Ringer’s solution + OT-7100 (i.e., the OT-7100 without DPCPX group) were significantly higher than those in the groups that received 5% acacia + Ringer’s solution, DPCPX + 5% acacia, or DPCPX + OT-7100 (Fig. 1).

Effects of DPCPX on anti-hyperalgesic effects of OT-7100 in the substance P model: Nociceptive thresholds in the paws in the group that received Ringer’s solution + OT-7100 (i.e., OT-7100 without DPCPX group) were significantly higher than those in the groups that received DPCPX + 5% acacia, or DPCPX + OT-7100 (Fig. 2).

Effects of DPCPX on anti-hyperalgesic effects of OT-7100 in the Bennett model: Nociceptive thresholds were significantly increased in the group that received Ringer’s solution + OT-7100 (i.e., OT-7100 without DPCPX group) in the injured paw on day 3 compared with the group that received DPCPX + OT-7100 (Fig. 3A). The nociceptive threshold in uninjured paw in the OT-7100 + DPCPX group was significantly lower than that in Ringer’s solution + OT-7100 on day 1, but there were no significant differences among the groups on other days (Fig. 3B).

Effects of DPCPX on anti-hyperalgesic effects of amitriptyline in the Bennett model: The nociceptive thresholds were significantly increased in both of the groups that received amitriptyline with and without DPCPX in the injured paw on day 5 (Fig. 4A). Differences were not significant in the uninjured paw (Fig. 4B).

Effects of the adenosine A$_2$ antagonist DMPX on anti-hyperalgesic effects of OT-7100 in the Bennett model: The nociceptive thresholds were significantly increased in
Effect of DPCPX on anti-hyperalgesic effect of OT-7100 in the Bennett model in rats (A, injured paw and B, uninjured paw: closed circle: Ringer’s solution + OT-7100, 10 mg/kg; open triangle: DPCPX, 0.01 pmol/animal + acacia; closed triangle: DPCPX, 0.01 pmol/animal + OT-7100, 10 mg/kg). DPCPX or Ringer’s solution was administered intrathecally 1 h before the oral administration of OT-7100 or acacia solution. The agents were administered for 3 consecutive days. Data are expressed as the mean ± S.D., n = 7 rats per group. **P<0.01 vs Ringer’s solution + OT-7100, Dunnett’s multiple comparison test.

Effect of DPCPX on anti-hyperalgesic effect of amitriptyline in the Bennett model in rats (A, injured paw and B, uninjured paw: closed circle: Ringer’s solution + amitriptyline, 30 mg/kg; open triangle: DPCPX, 0.01 pmol/animal + acacia; closed triangle: DPCPX, 0.01 pmol/animal + amitriptyline, 30 mg/kg). DPCPX or Ringer’s solution was administered intrathecally 1 h before the oral administration of amitriptyline or acacia solution. The agents were administered for 5 consecutive days. Data are expressed as mean ± S.D., n = 7 rats per group. **P<0.01 vs Ringer’s solution + amitriptyline, Dunnett’s multiple comparison test.
both of the groups that received OT-7100 with or without DMPX in the injured paw on day 3 (Fig. 5A). The nociceptive threshold in uninjured paw in the DMPX + OT-7100 group was significantly higher on day 3 than that in Ringer’s solution + OT-7100, but there were no significant differences among the groups on other days (Fig. 5B).

Anti-hyperalgesic effects of dipyridamole in the Bennett model: The decrease in nociceptive threshold in the injured paw was significantly inhibited from day 5 in the dipyridamole 100- and 300-mg/kg groups compared with the control group and the effect continued for 2 days after the final dose in the 300-mg/kg group. The decrease in nociceptive threshold in the injured paw was significantly inhibited from day 3 in the OT-7100 10-mg/kg group compared with the control group and the effect continued for 1 day after the final dose (Fig. 6A). The nociceptive threshold in the uninjured paw in the dipyridamole and OT-7100 groups remained unchanged (Fig. 6B).

SNL-induced mechanical hyperalgesia

The inhibitory effects of OT-7100 on peripheral neuropathic hyperalgesia as determined by changes in nociceptive threshold in the Chung model are shown in Fig. 7: A and B. The decrease in nociceptive threshold in the injured paw was significantly inhibited from day 7 in the 3-mg/kg group and from day 3 in the 10- and 30-mg/kg groups compared with the control group. The effect continued for 1 day after the final dose in the 3-mg/kg group and for 2 days after the final dose in the 10- and 30-mg/kg groups, after which no significant effects were observed. The nociceptive threshold in the uninjured paw remained unchanged.

Fos immunohistochemistry

In the control group 7 days after the CCI operation, Fos-LI neurons were observed in the ipsilateral dorsal horn, and they were most numerous in laminae 3 and 4 (24.02 ± 7.86) and less numerous in laminae 1 and 2 (16.72 ± 4.10) or deeper layers (16.72 ± 4.10). In the prophylactic and therapeutic groups, there was a significant decrease in the number of Fos-LI neurons, especially in laminae 3 and 4 of the dorsal horn, compared to that in the control group. A decrease in Fos-LI neurons of 45.8% (P<0.042) was seen on the ipsilateral side in the prophylactic group (13.70 ± 7.41) and decrease of 58.3% (P<0.015) was seen in the therapeutic group (10.30 ± 5.30), but there were no significant differences between the prophylactic and therapeutic groups (P>0.05). In the deeper layer on the ipsilateral side, there were no significant differences between the three groups (P>0.05) (Figs. 8 and 9). Fos-LI neurons were also observed in the contralateral dorsal horn. The number of positive neurons in laminae 1 and 2 was reduced in the both prophylactic (P<0.003) and therapeutic (P<0.027) groups compared to that in the control group.
Fig. 6. Time course of inhibitory effect of dipyridamole and OT-7100 on hyperalgesia measured by the noxious mechanical pressure-evoked hind paw withdrawal reflex in the Bennett model in rats (A, injured paw and B, uninjured paw: open circle: control; closed circle: dipyridamole, 100 mg/kg; open triangle: dipyridamole, 300 mg/kg; closed triangle: OT-7100, 10 mg/kg). The agent was orally administered once daily for 7 days. Data are expressed as mean ± S.D., n = 6 rats per group. *P<0.05, **P<0.01 vs Control, Dunnett’s multiple comparison test.

Fig. 7. Time course of inhibitory effect of OT-7100 on hyperalgesia measured by the noxious mechanical pressure-evoked hind paw withdrawal reflex in the Chung model in rats (A, injured paw and B, uninjured paw: open circle: control; closed circle: OT-7100, 3 mg/kg; open triangle: OT-7100, 10 mg/kg; closed triangle: OT-7100, 30 mg/kg). The agent was orally administered once daily for 14 days. Data are expressed as mean ± S.D., n = 7 rats per group. *P<0.05, **P<0.01 vs Control, Dunnett’s multiple comparison test.
No significant differences between any two groups were observed in other layers. Furthermore, the number of Fos-LI neurons in laminae 3 and 4 was equivalent on both sides in the prophylactic and therapeutic groups (Fig. 9).

**DISCUSSION**

In the present study, we studied the effects of OT-7100 on the inhibitory effects of adenosine on the contraction of guinea pig ileum by electrical stimulation and investigated if adenosinergic neurotransmission is involved in the antihyperalgesic activity of OT-7100 or not. We also studied the dose-response of the effect of OT-7100 on mechanical hyperalgesia using the Chung model of neuropathic pain. Furthermore, we investigated the effect of OT-7100 on neuronal activation in the spinal cord in the Bennett model using c-Fos expression as a marker.

The results of the present study indicate that the antihyperalgesic efficacy of OT-7100 is mediated, at least partly, by adenosinergic transmission at A_1 receptors. Increasing evidence has shown that adenosine plays a role in the modulation of nociceptive processing since the original observations in which methylxanthine adenosine analogs decreased nociceptive thresholds in rats (24). Adenosine, adenosine-receptor agonists, or adenosine-metabolism inhibitors administered intrathecally reduced inflammatory thermal hyperalgesia in rats (25), tactile allodynia in a rat model of neuropathic pain (13, 26), or nociceptive responses in tail flick and hot plate tests in mice (27). The anti-hyperalgesic effects of adenosine or adenosine analogues have also been reported in human studies. Intrathecally administered adenosine attenuated experimental pain induced by coldness, ischemia, or inflammation in healthy volunteers (28). Systemically or intrathecally administered adenosine also reduced spontaneous pain and tactile hyperalgesia in patients with chronic neuropathic pain (29, 30). In these papers, A_1 subtype of adenosine receptors has been suggested to participate in the anti-nociceptive effect of adenosine. It has also been reported that circulating and cerebrospinal fluid adenosine levels are reduced in patients with neuropathic pain compared with patients with nervous system lesion without pain, patients with non-neuropathic pain, and control subjects (15). Based on these findings, it is reasonable to assume that enhancing the effect of adenosine systemically or locally in the target organs results in alleviation of acute inflammatory pain or pain associated with neuropathy. Although we have not confirmed the augmentation of adenosine’s effects by OT-7100 in the animal models in which OT-7100 showed anti-nociceptive efficacy, OT-7100 enhanced the inhibitory effect of adenosine on the contraction of ileal longitudinal muscle by electrical stimulation in isolated guinea pig ileum preparations at the concentration of 0.3 – 3 μM. As the C_{max} of OT-7100, at which the compound showed efficacy in the Bennett model, was around 400 ng/mL (about 1 μM, in-house data), the concentration of OT-7100 at which enhancement of the inhibitory effect of adenosine was seen in in vitro
The efficacy of OT-7100 in yeast- or substance P-induced hyperalgesia or hyperalgesia induced by peripheral neuropathy in the Bennett model was significantly reversed by coadministration of the adenosine A1-receptor antagonist DPCPX. However, the efficacy of amitriptyline in the Bennett model was not reversed by DPCPX. These results suggest that the efficacy of OT-7100 in these animal models is mediated by adenosine A1 receptor, at least in part. It has been demonstrated that adenosine A2 receptors are also present in the dorsal horn of the spinal cord (31) and suggested to be involved in pain-processing (32). However, the efficacy of OT-7100 in the Bennett model was not reversed by coadministration of the adenosine A2-receptor antagonist DMPX, suggesting that A2 receptors are not involved in the effects of OT-7100. The reason for the increase in nociceptive threshold in uninjured paw in the DMPX + OT-7100 group is not clear. We have not confirmed the mechanisms by which OT-7100 enhances the effect of adenosine. Although OT-7100 definitely enhanced the effect of adenosine, OT-7100 showed only a weak affinity to adenosine A1 receptors (ED50 = 2.4 × 10^-4 M) and had weak antagonistic effects against an adenosine A1-receptor agonist in the in vitro study (S. Sato, unpublished data). These results suggest that OT-7100 enhances the effect of adenosine by increasing or maintaining the extracellular level of adenosine. OT-7100 showed no inhibition of adenosine-metabolizing enzymes such as adenosine deaminase and adenosine kinase at concentrations up to 10 μM (S. Sato, unpublished data). OT-7100 did not significantly inhibit the binding of 3H-nitrobenzylthioninosine (NBTI), which may represent NBTI-sensitive adenosine (nucleoside) transporter, to a membrane preparation from guinea pig cerebrum (S. Sato, unpublished data). However, there are subtypes of nucleoside transporters.

**Fig. 9.** Bar charts showing the number of c-Fos-LI neurons per section in the spinal cord of control rats or rats treated with OT-7100 (30 mg/kg, p.o.) prophylactically or therapeutically. Fos-LI expression was reduced in laminae 3, 4 in both prophylactic and therapeutic groups in the ipsilateral dorsal horn. Data are expressed as mean ± S.D., n = 4 or 5 rats per group. *P<0.05, **P<0.01 vs Control, Fisher’s PLSD test.
(33), suggesting the possibility that OT-7100 affects other subtypes of adenosine transporters. The result that OT-7100 affected neither motor nor cardiovascular functions in a general pharmacological study (in-house data) also suggests that OT-7100 affects substances other than adenosine transporters that are inhibited by known adenosine transport inhibitors, such as dipyridamole and drafizlenz, which were developed as agents for modulating cardiovascular function (34, 35). Dipyridamole significantly inhibited the nociceptive threshold in the injured paw compared with that in the control group when administered orally at 100 or 300 mg/kg per day for 7 days, but did not affect the nociceptive threshold in the uninjured paw. The time course of the effect of dipyridamole was similar to that of OT-7100.

It has also been reported that peripheral neuropathic pain is related to the sympathetic nervous system (1, 36). Another animal model of neuropathic pain, the rat spinal nerve ligation model (i.e., the Chung model), is thought to be a model of sympathetically maintained pain (SMP) because the hyperalgesia is attenuated by excising the sympathetic nerves (37, 38). The effect of OT-7100 was investigated in this model by measuring the nociceptive threshold to noxious pressure stimulation in the injured paw and in the uninjured paw by the Randall-Selitto method. The nociceptive threshold in the injured paw was reduced to a level comparable to that observed in the injured paw in the Bennett model at 2 weeks after surgery, and this level was maintained until the end of the experiment in the control group. OT-7100 significantly inhibited the decrease in nociceptive threshold in the uninjured paw compared with that in the control group when administered orally at 3, 10 or 30 mg/kg per day for 14 days, but did not affect the nociceptive threshold in the uninjured paw. Because OT-7100 inhibited the decrease in the nociceptive threshold only in the injured paw and had no effect on the uninjured paw, OT-7100 is considered to specifically inhibit peripheral neuropathic hyperalgesia. These results, taken together, suggest that OT-7100 is useful for the treatment of neuropathic pain including SMP in clinical use. It should be emphasized that this agent was not effective in normalizing the nociceptive threshold immediately after administration, but showed effects starting from day 3 of administration and that the effects persisted for 1 to 2 days after the end of administration.

The findings of the present study demonstrated that the administration of OT-7100 in the prophylactic and therapeutic groups significantly decreased the number of Fos-LI neurons in the spinal dorsal horn in the Bennett model. This result indicates that the mechanism for the improvement of hyperalgesia in the Bennett model involves the primary afferent system and/or secondary neurons in the dorsal horn. Fos-LI neurons were also observed in the contralateral dorsal horn. This contralateral expression may be partly due to direct sensory input from the opposite paw that is bearing more weight (39) in order to compensate for the injured paw. Adenosine, adenosine receptor agonists, or adenosine metabolism inhibitors administered intrathecally reduced tactile allodynia in a rat model of neuropathic pain (13, 26). In addition, an adenosine analogue, adenosine kinase, and deaminase inhibitor reduced carrageenin-induced c-Fos expression in the spinal dorsal horn (40, 41). Overall, it is possible that the attenuation in spinal c-Fos expression is a consequence of the enhancement of adenosine’s action in the spinal dorsal horn.

In conclusion, OT-7100 inhibited mechanical hyperalgesia in inflammatory and peripheral neuropathic pain models and the effect of OT-7100 in these animal models was mediated by adenosine. Furthermore, dorsal horn neurons and/or the primary afferent system were considered as the site of action of OT-7100, since it suppressed c-Fos expression in dorsal horn neurons in the Bennett model. Although further studies are needed to bridge the in vitro adenosine-enhancing effect of OT-7100 with the antinociceptive efficacy and to elucidate mechanisms by which adenosine attenuates neuropathic pain, the results of this study suggest that agents such as OT-7100 should prove useful for the treatment of chronic neuropathic pain.

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