AS1069562, the (+)-Isomer of Indeloxazine, Exerts Analgesic Effects in a Rat Model of Neuropathic Pain with Unique Characteristics in Spinal Monoamine Turnover

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ABSTRACT

AS1069562 [(R)-2-[(1H-inden-7-yl)oxy]methyl][morpholine monobenzenesulfonate] is the (+)-isomer of indeloxazine, which had been used clinically for the treatment of cerebrovascular diseases with multiple pharmacological actions, including serotonin (5-HT) and noradrenaline (NE) reuptake inhibition. Here we investigated the analgesic effects of AS1069562 in a rat model of chronic constriction injury (CCI)-induced neuropathic pain and the spinal monoamine turnover. These effects were compared with those of the antidepressants duloxetine and amitriptyline. AS1069562 significantly elevated extracellular 5-HT and NE levels in the rat spinal dorsal horn, although its 5-HT and NE reuptake inhibition was much weaker than that of duloxetine in vitro. In addition, AS1069562 increased the ratio of the contents of both 5-HT and NE to their metabolites in rat spinal cord, whereas duloxetine slightly increased only the ratio of the content of 5-HT to its metabolite. In CCI rats, AS1069562 and duloxetine significantly ameliorated mechanical allodynia, whereas amitriptyline did not. AS1069562 and amitriptyline significantly ameliorated thermal hyperalgesia, and duloxetine tended to ameliorate it. Furthermore, AS1069562, duloxetine, and amitriptyline significantly improved spontaneous pain–associated behavior. In a gastric emptying study, AS1069562 affected gastric emptying at the same dose that exerted analgesia in CCI rats. On the other hand, duloxetine and amitriptyline significantly reduced gastric emptying at lower doses than those that exerted analgesic effects. These results indicate that AS1069562 broadly improved various types of neuropathic pain–related behavior in CCI rats with unique characteristics in spinal monoamine turnover, suggesting that AS1069562 may have potential as a treatment option for patients with neuropathic pain, with a different profile from currently available antidepressants.

Introduction

Neuropathic pain is defined as “pain arising as a direct consequence of a lesion or disease affecting the somatosensory system” (Treede et al., 2008). Despite the existence of recent evidence-based treatment recommendations, treatment of neuropathic pain remains difficult. Antidepressants such as the serotonin (5-HT) and noradrenaline (NE) reuptake inhibitor duloxetine and the tricyclic antidepressant amitriptyline are the first-line agents recommended for the treatment of neuropathic pain. The analgesic effects of these antidepressants are based primarily upon the enhancement of 5-HT and NE neurotransmission, which involves endogenous analgesic mechanisms mediated by the descending inhibitory pain pathways in the brain and spinal cord (Thor et al., 2007). However, although the clinical effects of antidepressants have been well established in neuropathic pain, their analgesic efficacies are still incomplete and there exist non-responder patients. Further, gastric side effects such as nausea and vomiting are frequently observed and may result in insufficient dose escalation to achieve efficacy and in early discontinuation of the medication (Bymaster et al., 2005; Rosenzweig-Lipson et al., 2007). As such, more efficacious and safer agents with different profiles from those of currently available antidepressants are urgently needed.

Indeloxazine is considered to be a cerebral metabolic enhancer with cerebral-activating properties; that is to say, it shows facilitatory effects on learning behavior and protective effects against cerebral injuries by increasing ATP and glucose levels in the brain (Harada et al., 1987; Shimizu-Sasamata et al., 1991). This drug had been used in Japan and South Korea for the treatment of psychiatric symptoms associated with cerebrovascular disease, namely poststroke depression, emotional disturbance, and reduced vomiting (Yamamoto, 1990). In an in vitro study, indeloxazine has been shown to have an affinity for 5-HT and NE transporter sites, with...
potency similar to amitriptyline. The compound has also been shown to inhibit 5-HT and NE reuptake in rat cerebral cortex (Yamaguchi et al., 1998). In in vivo neurochemical studies, indeloxazine has been shown to produce a variety of pharmacological effects: increase in extracellular levels of 5-HT and NE in rat prefrontal cortex (Yamamoto et al., 1993), increase in extracellular levels of acetylcholine in rat frontal cortex via indirect effect on endogenous 5-HT and 5-HT4 receptor pathways (Yamaguchi et al., 1997), and increases in ATP and glucose levels in mouse brain (Harada et al., 1987).

In animal behavior studies, antidepressant-like effects, protective effects against cerebral ischemia, and the facilitation of passive avoidance learning behavior have also been reported (Shimizu-Sasamata et al., 1991). AS1069562, the (+)-isomer of indeloxazine, also has demonstrated inhibitory effects on 5-HT and NE reuptake in rat brain, a facilitatory effect on spontaneous electroencephalogram in rats, and a facilitatory effect on passive avoidance learning behavior in rats (Shimizu-Sasamata et al., 1993). Here we evaluated the effects of AS1069562 on spinal 5-HT and NE extracellular concentrations and turnover rates, various types of pain-related behaviors in a rat model of chronic constriction injury (CCI)-induced neuropathic pain, and adverse events related to gastric function. The results were compared with those of the currently available antidepressants duloxetine and amitriptyline.

Materials and Methods

\((R)-2\)-(1H-Inden-7-yl oxymethyl)morpholine monobenzenesulfonate (AS1069562) (Fig. 1) and duloxetine were synthesized at Astellas Pharma Inc. (Ibaraki, Japan). Both chemicals were used in their hydrochloride salt form. Amitriptyline and reserpinatoxin (RTX) were purchased from Sigma-Aldrich (St. Louis, MO). For in vivo studies, AS1069562, duloxetine, and amitriptyline were suspended in distilled water. These compounds were administered orally at an administration volume of 5 ml/kg. RTX was suspended in 10% ethanol, 10% Tween 80, and 80% saline and was administered subcutaneously at an administration volume of 5 ml/kg. RTX was suspended in 10% ethanol, 10% Tween 80, and 80% saline and was administered subcutaneously in 5 ml/kg. Drug concentrations were measured in terms of base free. AS1069562 and amitriptyline were administered orally 1 hour before behavioral testing or sampling, and duloxetine was administered orally 3 hours before behavioral testing or sampling, and RTX was administered subcutaneously 48 hours before behavioral testing.

Animals. Male Sprague-Dawley rats (weight range: 156–387 g; Japan SLC, Hamamatsu, Japan) were used for all in vivo experiments. Animals were group-housed under a 12-hour light/dark cycle (light on: 7:30 AM to 7:30 PM) at 23 ± 1°C, with free access to food and water. All animal experimental procedures were approved by the Committee for Animal Experiments of Astellas Pharma Inc. All efforts were made to minimize the number of animals used and their suffering.

Cell Culture. Human embryonic kidney (HEK) 293 cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (37°C, 5% CO2). HEK293 cells stably expressing human or rat 5-HT transporter were generated by transfection with a pcDNA 3.1 (+) expression vector encoding the appropriate 5-HT transporter cDNA. Lipopectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) was used to create stable transfections, and 800 μg/ml G-418 was used as a selection agent. Positive clones that showed high reuptake activity were used in all in vitro studies. HEK293 cells stably expressing NE and dopamine (DA) transporters were constructed using similar procedures.

Uptake Assay. HEK293 cells expressing recombinant human or rat 5-HT, NE, or DA transporter were plated onto 96-well assay plates at a density of 2 × 10⁴ cells/well and cultured overnight in the incubator (37°C, 5% CO2). For measurement of uptake of [3H]5-HT, [3H]NE, or [3H]DA, the culture medium was removed from the 96-well plates, the cells were washed once with Krebs-Ringer-HEPES (KRH) buffer (125 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO4, 1.2 mM KH2PO4, 25 mM HEPES, 5.6 mM glucose, 1 mM ascorbic acid, 0.1 mM parglyine; pH 7.3) containing 0.1% bovine serum albumin, and samples were then preincubated at 25°C for 10 minutes with KRH buffer containing various concentrations of drugs. Subsequently, the cells were incubated for 20 minutes at 25°C with KRH buffer containing ~25 nM [3H]-5-HT, [3H]-NE, or [3H]-DA. The incubation was terminated by aspiration of the incubation medium and rapid washing of the cells once with ice-cold KRH buffer. Thereafter the cells were lysed in 15 μl of 0.1 N NaOH, and 100 μl of Microscint-PS (PerkinElmer Life and Analytical Sciences, Boston, MA) was added into the wells. The radioactivity of the cell lysates was determined by scintillation counting.

Binding Assay. The binding assay was conducted using cell membranes expressing human recombinant 5-HT, NE, or DA transporter (11.3, 6.0, or 12.0 μg of protein, respectively) (PerkinElmer Life and Analytical Sciences). For 5-HT transporter binding assay, [benzene ring-3H](N)-imipramine hydrochloride (1.4 nM; PerkinElmer Life and Analytical Sciences) and competitor (imipramine hydrochloride; Sigma-Aldrich) were incubated at 25°C for 30 minutes in 50 mM Tris-HCl (pH 7.4) buffer containing 100 mM NaCl. For NE transporter binding assay, [N-methyl-3H]nisoxetine hydrochloride (0.45 nM; PerkinElmer Life and Analytical Sciences) and competitor (desipramine hydrochloride; Sigma-Aldrich) were incubated at 4°C for 60 minutes in 50 mM Tris-HCl (pH 7.4) buffer containing 120 mM NaCl and 5 mM KCl. For DA transporter binding assay, [N-methyl-3H]WIN35,428 ([1-2-(b-carbomethoxy-3-β-(4-fluorophenyl)tropane] (1.7 nM; PerkinElmer Life and Analytical Sciences) and competitor (GBR12909 dihydrochloride [1-(2-bis[4-fluorophenyl]methoxyethyl)-4-(3-phenylpropyl)piperazine]; Sigma-Aldrich) were incubated at 4°C for 120 minutes in 50 mM Tris-HCl (pH 7.4) buffer containing 120 mM NaCl and 5 mM KCl. To determine nonspecific binding, 100 μM imipramine hydrochloride, desipramine hydrochloride, or GBR12909 dihydrochloride was used for 5-HT, NE, or DA assay, respectively. The incubated mixtures were filtered using a cell harvester. The filter paper was rinsed three times with 50 mM Tris-HCl (pH 7.4) buffer. Atomlight (PerkinElmer Life and Analytical Sciences) was added, and the radioactivity of the filter paper was determined via scintillation counting. Specific binding was defined as a portion of total binding that was replaced by 100 μM imipramine hydrochloride, desipramine hydrochloride, or GBR12909 dihydrochloride for 5-HT, NE, or DA assay, respectively. The dissociation constant (Kd) and binding site density (Bmax) were calculated via Scatchard analysis, whereas IC50 values were calculated via nonlinear regression analysis. The values of the apparent equilibrium dissociation constant of inhibitors (Ki) were calculated using the method of Cheng and Prusoff (1973).

Spinal Dorsal Horn Microdialysis. The spinal dorsal horn microdialysis was performed in accordance with the method described.
by Kawamata et al. (2002). Dialysis probes were constructed from a 1-cm length of dialysis fiber (DM-22; Eicom Co., Kyoto, Japan), which was coated with a thin layer of epoxy glue (Devcon Co., Danvers, MA) along the whole length, except for a 2-mm region in the middle. To make the fiber firm enough for implantation, a Nichrome-Formvar wire with a 78-μm internal diameter (A-M Systems Inc., Everett, WA) was passed through the fiber. One end of the fiber was attached to 2-cm polyethylene catheters (PE-10; Becton Dickinson, Franklin Lakes, NJ), and the end of the polyethylene catheters was then attached to an 8-cm polytetrafluoroethylene tube (JT-10; Eicom). The other end of the fiber was attached to a pin. Rats were anesthetized with sodium pentobarbital and incised along the dorsal midline from T2 to L2. The lateral surfaces of vertebrae L1 were exposed, and bilateral holes were carefully drilled through the bone using a 25-gauge needle, exposing the spinal cord laterally at the level of the dorsal horn. A dialysis probe was placed through the holes by hand, passing transversely through the dorsal spinal cord. At this point, the dialysis tubing, the initial portions of the PE-10 tubes, and the exposed surface of L1 were encased in dental acrylic. The pin was removed from the end of the fiber, and the 2-cm polyethylene catheter and 8-cm polytetrafluoroethylene tube were attached. The two distal ends of the probe were tunneled subcutaneously and externalized through the skin in the neck region. The experiments were performed 18–24 hours after the implantation of the dialysis probe. After a recovery period, animals showing any signs of limb paralysis or impaired movement were excluded from the study. The dialysis probe was perfused with Ringer’s solution (140 mM NaCl, 4.0 mM KCl, 1.26 mM CaCl2, and 1.15 mM MgCl2) at a constant flow rate of 1 μl/min. The outflow was collected in a sample tube every 30 minutes. Five rats were used in each group. The samples were frozen at −80°C until analysis. Extracellular 5-HT and NE levels in the spinal cord were measured using high-performance liquid chromatography (HPLC) with an electrochemical detection system (HTEC-500; Eicom).

Contents of Monoamines and Their Metabolites in Spinal Cord. Rat lumbar spinal cord samples were collected and homogenized with 0.2 M perchloric acid and 100 μM EDTA. After centrifugation (1500g, 10 minutes, 4°C), the supernatant was separated and stored at −80°C until analysis. Five rats were used in each group. The contents of monoamines (5-HT and NE) and their metabolites (5-hydroxyindolacetic acid (5-HIAA) and 4-hydroxy-3-methoxyphenylglycol (MHPG), respectively) in the samples were quantified using HPLC with an electrochemical detection system (ECD-300; Eicom).

Pharmacokinetics Study. Plasma samples were collected from rats at 0.25, 0.5, 1, 4, and 8 hours after oral administration of 10 mg/kg AS1069562 or 0.5, 1, 2, 3, 4, and 9 hours after oral administration of 10 mg/kg duloxetine. Plasma samples were collected from rats at 1 hour after oral administration of 10 mg/kg AS1069562 or 3 hours after oral administration of 10 mg/kg duloxetine. Three rats were used in each group. Plasma and brain samples were stored at −20°C until use. Brain samples were homogenized with 100 mM sodium-potassium phosphate buffer (pH 7.4). Plasma and brain samples were subjected to protein precipitation with acetonitrile. After centrifugation (1500g, 10 minutes, 4°C), the supernatant was separated and used for analysis. The concentration of AS1069562 in the samples was quantified using HPLC (Prominence UFLC; Shimadzu Corp., Kyoto, Japan) coupled with a triple quadrupole mass spectrometer (API4000; Applied Biosystems, Rockville, MD).

CCI Surgery. CCI surgery was performed in accordance with the method of Bennett and Xie (1988). Animals were anesthetized with pentobarbital, and the left common sciatic nerve was exposed at the level of the midtigh through the biceps femoris. Proximal to the sciatic trifurcation, the nerve was separated from the adhering tissue, and four loose ligatures of 4-0 chromic gut were applied around the nerve with ~1 mm of spacing between them. The incision was then closed within the muscle and skin layers. The sham group of animals was subjected to all surgical operations, but the nerve ligature was not applied.

Mechanical Allodynia (Van Frey Hair Test). The cutaneous nociceptive threshold was measured by modification of a previously reported method (Chapman et al., 1994). In brief, rats were placed in a test cage with a metal-mesh floor, and von Frey filaments (0.4, 0.7, 1.2, 2.0, 3.6, 5.5, 8.5, and 15.1 g) were applied to the plantar surface of the left hind paw. A positive or negative response was defined as the paw withdrawal response from the pressure of a filament or the lack of a response within 6 seconds, respectively. The 2.0-g force filament was applied first. If a positive response to a given filament occurred, the next-smaller filament was then applied. If a negative response occurred, the next-larger filament was applied. The test continued until four responses were collected after the first change in response, and then the paw withdrawal threshold was converted to the cutaneous nociceptive threshold using an adaptation of the Dixon up-down paradigm. On the day before drug evaluation, the rats underwent withdrawal threshold measurement and were allocated to groups to minimize the differences in average threshold among groups. Eight to 11 rats were used in each group. Drug evaluation was performed at 2 weeks after CCI surgery, when mechanical allodynia reached almost the maximum. All behavioral responses were measured in a blind manner.

Thermal Hyperalgesia (Plantar Test). Thermal hyperalgesia was assessed using the plantar test (model 7370; Ugo Basile, Varese, Italy) and a modified method of Hargreaves et al. (1988). In brief, the rats were habituated to an apparatus consisting of individual perspex boxes on an elevated glass table. A mobile radiant heat source was located under the table and focused on the hind paw, and the paw withdrawal latencies were defined as the time taken by the rat to remove its hind paw from the heat source. The cutoff point was set at 15 seconds to prevent tissue damage. The apparatus was calibrated to give a paw withdrawal latency of ~10 seconds in naive rats. On the day before drug evaluation, the rats underwent withdrawal latency measurement and were allocated to groups to minimize the differences in average latency among groups. Eight to 11 rats were used in each group. Drug evaluation was performed at 2 weeks after CCI surgery, when thermal hyperalgesia reached almost the maximum. All behavioral responses were measured in a blind manner.

Spontaneous Pain–Associated Limb Movement. Spontaneous pain–associated limb movement was measured as described previously (Kawasaki-Yatsugi et al., 2012). Briefly, a small polytetrafluoroethylene-coated columnar magnet (1 mm in diameter, 3 mm long) (SCT-MAG-TF; Neuroscience Inc., Tokyo, Japan) was implanted into the s.c. space of the left hind limb dorsum under isoflurane anesthesia. Two or more days after the implantation, the animals were acclimated to dim light (~20 lux) for at least 20 minutes and then placed inside the test chamber (28 cm in diameter, 25 cm in height; NS-SC10R; Neuroscience Inc.), which was surrounded by a round coil of 7000 turns. The chamber was set to dim light and constant room temperature (22 ± 2°C). Measurements began after a 5-minute acclimation period inside the test chamber. Voltage within the coil was induced by a change in the electromagnetic field associated with the movement of the magnet implanted in the hind limb. Limb movements were automatically detected as spike waveforms and counted using the MicroAct analyzing software (NS-SC-S100; Neuroscience Inc.). Analysis parameters for the limb movement–related waveforms were set at a frequency range of 2.5–20 Hz, with a threshold of 0.01 V, minimum duration of 0.09 second, and minimum gap duration of 0.03 second. CCI rats exhibit frequent aberrant asymmetric movement, such as lifting/guarding, flinching/shaking, and licking, which are considered indicative of spontaneous pain (Mogil and Crager, 2004). The spontaneous pain–associated limb movement frequencies in 30 minutes were determined by automated measurement. On the day before drug evaluation, the rats underwent spontaneous pain–associated limb movement frequency measurement and were allocated to groups to minimize the differences in average frequency among groups. Nine to 12 rats were used in each group. Drug evaluation was performed at 3 weeks after CCI surgery, when spontaneous pain–associated limb movement frequency reached almost the maximum.

Gastric Emptying Ability. Gastric emptying of liquids was assessed by the phenol red method. Rats were fasted from the evening prior to the day of assessment, and 1.5% methylcellulose

374 Murai et al.
solution containing 0.01% phenol red was orally administered at an administration volume of 1.5 ml/animal immediately after oral drug administration. Rat stomachs were collected at 15 minutes after phenol red administration. Six rats were used in each group. Stomach samples were homogenized with 0.1 N NaOH and subjected to protein precipitation with trichloroacetic acid. The homogenates were centrifuged at 2000g for 10 minutes at 4°C. The supernatant was separated via high-speed centrifugation at 20,000g for 10 minutes at 4°C and used for analysis. The absorbance of phenol red extracted from individual stomach samples was read at 560 nm by a spectrophotometer (SpectraMax Plus 384; Molecular Devices, Sunnyvale, CA). Gastric emptying ability was evaluated as the percentage of change in dye excreted from stomach.

**Locomotor Activity.** Locomotor activity was measured using an activity monitoring system (Supermex; Muromachi Kikai, Tokyo, Japan). Rats were placed into an open field, and the activity count was recorded every 5 minutes for 60 minutes. Eight rats were used in each group.

**Rotarod Performance Assessment.** Motor coordination was measured using an accelerating rotarod apparatus (LE8500; Panlab, Barcelona, Spain) set to accelerate from 4 to 40 rpm over 5 minutes. Each rat was subjected to three training sessions. Rats that remained on the rod for >90 seconds were used for the test session and allocated to groups to minimize the differences in average maximum performance times in the training sessions among groups. Eight rats were used in each group. In the test session, retention time was measured twice, and the mean was adopted as a data point for each animal.

**TABLE 1**

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<th>IC$_{50}$ values of AS1069562 and duloxetine for 5-HT, NE, and DA reuptake activities</th>
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<td><strong>Human</strong></td>
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**Statistical Analysis.** Data were expressed as the mean and 95% confidence interval for uptake assay; as the mean ± S.D. for pharmacokinetic profiles; and as the mean ± S.E.M. for binding affinity, extracellular monoamine levels, concentrations of monoamines and their metabolites, CCI model, gastric emptying ability, locomotor activity, and rotarod performance assessment. The significance of differences between two groups was assessed using Student’s $t$ test, while that among more than two groups was assessed using Dunnett’s multiple-comparisons tests. $P < 0.05$ was considered significant.

![Fig. 2](https://example.com/fig2.png)

**Fig. 2.** Effects of AS1069562 and duloxetine on extracellular 5-HT (A) and NE (B) levels in rat spinal dorsal horns. AS1069562 and duloxetine were orally administered 1 and 3 hours, respectively, before sampling. Each bar represents the mean ± S.E.M. for five rats. *$P < 0.05$; ***$P < 0.001$ by Dunnett’s test vs. vehicle-treated group.
Results

Inhibitory Effects of AS1069562 and Duloxetine on 5-HT, NE, and DA Reuptake Activities. AS1069562 inhibited 5-HT and NE reuptake activities with IC_{50} values of 0.35 and 3.3 µM, respectively, in HEK293 cells expressing recombinant human transporters, and of 0.38 and 4.0 µM in HEK293 cells expressing recombinant rat transporters. The potencies of AS1069562 for inhibiting these reuptakes were lower (~9-fold for 5-HT and ~310-fold for NE in rats) than those of duloxetine. AS1069562 did not inhibit DA reuptake activities. The results are summarized in Table 1.

Binding Affinity of AS1069562 for 5-HT, NE, and DA Transporters. AS1069562 had affinities for human 5-HT, NE, and DA transporters with respective K_i values of 0.040 ± 0.0032, 4.4 ± 0.15, and 4.8 ± 0.21 µM.

Effects of AS1069562 and Duloxetine on Extracellular Monoamine Levels in Rat Spinal Dorsal Horn. AS1069562 orally administered at 10 and 30 mg/kg significantly elevated extracellular 5-HT and NE levels in rat spinal dorsal horn. Duloxetine orally administered at 30 mg/kg also tended to elevate 5-HT and NE levels. AS1069562 elevated extracellular 5-HT levels in rat spinal dorsal horn to ∼192% of that of duloxetine and elevated extracellular NE levels to ∼43% of that of duloxetine (Fig. 2).

Effects of AS1069562 and Duloxetine on Contents of Monoamines and Their Metabolites in Rat Spinal Cords. Whereas AS1069562 orally administered at 10 and

<table>
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<th>Contents of Monoamine or Metabolite Following Treatment With:</th>
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<th>AS1069562</th>
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<tr>
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<td>3 mg/kg</td>
<td>10 mg/kg</td>
<td>30 mg/kg</td>
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<tr>
<td>5-HT</td>
<td>590 ± 61</td>
<td>629 ± 30</td>
<td>771 ± 41*</td>
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<tr>
<td>5-HIAA</td>
<td>285 ± 26</td>
<td>301 ± 12</td>
<td>233 ± 18</td>
</tr>
<tr>
<td>NE</td>
<td>293 ± 3.3</td>
<td>276 ± 15</td>
<td>359 ± 25*</td>
</tr>
<tr>
<td>MHPG</td>
<td>25.2 ± 3.1</td>
<td>24.8 ± 3.5</td>
<td>15.3 ± 1.4*</td>
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*P < 0.05; **P < 0.01; ***P < 0.001 by Dunnett’s test vs. vehicle-treated group.
30 mg/kg significantly increased 5-HT and NE contents in rat spinal cord homogenate, duloxetine orally administered at doses up to 30 mg/kg did not increase 5-HT or NE contents. In the same samples, contents of 5-HIAA and MHPG, which are metabolites of 5-HT and NE, respectively, were reduced by AS1069562 and duloxetine administration (Table 2). Furthermore, AS1069562 orally administered at 10 and 30 mg/kg significantly and dramatically increased the ratios of both 5-HT to 5-HIAA and NE to MHPG. In contrast, 30 mg/kg duloxetine significantly but slightly increased only the ratio of 5-HT to 5-HIAA (Fig. 3). The results of this study indicate that AS1069562 has a unique effect on spinal 5-HT and NE turnover, not only inhibiting 5-HT and NE metabolism but also potentiating 5-HT and NE increases in the spinal cord.

Pharmacokinetic Profiles of AS1069562 and Duloxetine in Rats. The plasma concentrations of AS1069562 reached levels of 16 ng/ml (60 nM) at 1 hour after oral administration of 10 mg/kg in rats (Fig. 4). The brain levels of AS1069562 at 1 hour after oral administration of 10 mg/kg were 612 ng/g (2286 nM), and the brain-to-plasma concentration ratio was 36. The plasma concentrations of duloxetine peaked at 15 ng/ml (45 nM) 3 hours after oral administration of 10 mg/kg in rats (Fig. 4). The brain levels of duloxetine at 3 hours after oral administration of 10 mg/kg were 283 ng/g (848 nM), and the brain-to-plasma concentration ratio was 22.

Analgesic Effects of AS1069562, Duloxetine, and Amitriptyline on Mechanical Allodynia, Thermal Hyperalgesia, and Spontaneous Pain Behavior in CCI Rats. To determine the analgesic effects of AS1069562, duloxetine, and amitriptyline on mechanical allodynia, thermal hyperalgesia, and spontaneous pain in a rat model of neuropathic pain, we performed the von Frey test, plantar test, and spontaneous pain–associated limb movement measurement. Withdrawal threshold, an index of mechanical allodynia, and
withdrawal latency, an index of thermal hyperalgesia, were significantly decreased, and limb movement frequency, an index of spontaneous pain behavior, was significantly increased in CCI rats.

AS1069562 significantly ameliorated mechanical allodynia at an oral dose of 30 mg/kg, with a maximum possible effect (MPE) of 70% (Fig. 5A). Duloxetine also significantly improved mechanical allodynia in this model at a dose of 30 mg/kg, with an MPE of 54% (Fig. 5B), whereas amitriptyline did not show significant improvement even at doses up to 100 mg/kg (Fig. 5C).

AS1069562 significantly ameliorated thermal hyperalgesia at an oral dose of 30 mg/kg, with an MPE of 112% (Fig. 6A). Amitriptyline also significantly improved thermal hyperalgesia in this model at a dose of 30 mg/kg, with an MPE of 54% (Fig. 6B), whereas duloxetine did not show significant improvement even at doses up to 100 mg/kg (Fig. 6D).

To assess the involvement of C-fiber in mechanical allodynia and thermal hyperalgesia in CCI rats, we examined the effects of RTX, an ultrapotent analog of capsaicin. Desensitization of C-fiber with systemic RTX significantly and completely improved thermal hyperalgesia at an s.c. dose of 0.3 mg/kg (Fig. 6D), and also significantly but only partially improved mechanical allodynia (Fig. 5D). These results indicate that hyperexcitability of C-fiber plays a major role in thermal hyperalgesia and a partial role in mechanical allodynia in CCI rats.

AS1069562 significantly ameliorated spontaneous pain behavior at oral doses of 10 and 30 mg/kg, with an MPE of 80% (Fig. 7A). Further, duloxetine (30 mg/kg, with an MPE of 85%) and amitriptyline (30 and 100 mg/kg, with an MPE of 87%) also significantly recovered spontaneous pain behavior in this model (Fig. 7, B and C).

Effects of AS1069562, Duloxetine, and Amitriptyline on Gastric Emptying in Rats. AS1069562 did not affect gastric emptying ability at oral doses up to 10 mg/kg, and significant reduction (46%) in the amount of dye excreted from stomach was observed at 30 mg/kg (Fig. 8A). In contrast, duloxetine and amitriptyline significantly decreased the amount of dye excreted from the stomach by 22% at 3 mg/kg and by 48% at 10 mg/kg, respectively, and duloxetine induced a pronounced decrease in excretion (100%) at 30 mg/kg (Fig. 8, B and C).

Effects of AS1069562, Duloxetine, and Amitriptyline on Locomotor Activity in Rats. AS1069562 had no significant effect on locomotor activity, an index of horizontal and vertical movements, at oral doses up to 100 mg/kg. In contrast, duloxetine and amitriptyline significantly reduced locomotor activity at 100 and 300 mg/kg, respectively (Table 3).
Effects of AS1069562, Duloxetine, and Amitriptyline on Rotarod Performance in Rats. AS1069562, duloxetine, and amitriptyline had no significant effect on rotarod performance, an index of ataxia or sedation, at oral doses up to 100, 100, and 300 mg/kg, respectively (Table 3).

Discussion

Here we demonstrated that AS1069562 inhibited 5-HT and NE reuptake activities in human and rat transporter-expressing cells with lower potencies than duloxetine. Of note, AS1069562 elevated extracellular 5-HT levels in rat spinal dorsal horn to ∼192% of that of duloxetine, and extracellular NE levels to ∼43% of that of duloxetine. Our pharmacokinetic study demonstrated only a 2.7-fold difference in molar brain concentration after an oral dose of 10 mg/kg between AS1069562 and duloxetine. These results suggest that the elevation of 5-HT and NE by AS1069562 is not simply due to its reuptake inhibition.

Next we demonstrated that AS1069562 increased 5-HT and NE contents and decreased contents of 5-HIAA and MHPG, which are the respective metabolites of 5-HT and NE in rat spinal cord. In contrast, duloxetine only decreased 5-HIAA and MHPG contents. In the spinal dorsal horn, endogenous 5-HT and NE have crucial influences on pain processing (Millan, 2002). Upon termination of their synaptic transmission, 5-HT and NE are taken up again or metabolized to 5-HIAA and MHPG, respectively. Our observations here indicate that AS1069562 not only reduced 5-HT and NE metabolism, which was also observed in duloxetine, but also potentiated 5-HT and NE increase in the spinal cord, which was a unique effect of AS1069562. While the former effects might have been caused by 5-HT and NE reuptake inhibition, since duloxetine also had the same effects, the latter effects might have been caused by antagonizing autoreceptors. AS1069562 has an affinity for 5-HT1B receptor at almost the same potency as 5-HT transporter (Murai et al., submitted for publication). Given that 5-HT1B receptors have been reported to act as autoreceptors, inhibiting the release of 5-HT at serotonergic nerve terminals (De Groote et al., 2003; Pytlak et al., 2011), AS1069562 may enhance release of 5-HT via its effect on 5-HT1B receptors at doses similar to those used to achieve 5-HT transporter inhibition. Indeed, ASP1069562 did increase 5-HT content in the spinal cord in the present study. In contrast, duloxetine has been reported to have low affinity for 5-HT1B receptors, with a Ki value of 4.0 μM, which was ∼5000 times lower than that of 5-HT transporter (Ki value: 0.0008 μM) (Bymaster et al., 2001), and in the present study, duloxetine had no effect on 5-HT content in the spinal cord. Taken together, the results suggest that AS1069562 is anticipated to exert beneficial analgesic effects based on its unique effect on 5-HT and NE turnover in the spinal cord.

We demonstrated that AS1069562 significantly improved both mechanical allodynia and thermal hyperalgesia in CCI rats. Duloxetine significantly improved mechanical allodynia and tended to ameliorate thermal hyperalgesia. In contrast, amitriptyline significantly ameliorated only thermal hyperalgesia. In addition, desensitization of C-fiber with systemic RTX suppressed CCI-induced thermal hyperalgesia completely but suppressed CCI-induced mechanical allodynia.
only partially. Previous studies reported that systemic treatment with RTX or capsaicin prevented and suppressed CCI-induced thermal hyperalgesia (Meller et al., 1992; Hama, 2002; Gaus et al., 2003), which is consistent with our results. These present and previous findings suggest that hyperexcitability of C-fiber plays a major role in thermal hyperalgesia and is partially responsible for mechanical allodynia in CCI rats. Taken together, our data indicate that AS1069562 inhibits hypersensitivity triggered by both C-fibers and non-C-fibers (A-fibers). In contrast, amitriptyline only inhibits C-fiber-triggered hypersensitivity, suggesting that AS1069562 may have a broader analgesic effect than amitriptyline due to the drug’s inhibition of both C-fiber- and A-fiber-triggered hypersensitivity.

In addition, AS1069562 significantly improved limb movement frequency, which is an index of spontaneous pain, in CCI rats at oral doses of 10 and 30 mg/kg. Although AS1069562 inhibited rat 5-HT reuptake activities by ~9-fold and NE reuptake activities by ~300-fold, representing lower potency than duloxetine, AS1069562 showed analgesic effects in a rat model of neuropathic pain at a similar dose to duloxetine. However, the reason that AS1069562 shows analgesic effects at a similar dose to duloxetine with lower potency of 5-HT and NE reuptake inhibition than duloxetine remains unclear. Recently we found that AS1069562 not only inhibits NE and 5-HT reuptake but also has an affinity for 5-HT1A and 5-HT3 receptors at almost the same potency as 5-HT reuptake inhibition, but in contrast, no appreciable affinity for the 5-HT2A, 5-HT2C, 5-HT4, 5-HT5A, 5-HT6, and 5-HT7 receptors. Further in vivo mechanistic study suggests that 5-HT1A agonism and 5-HT3 antagonism could be involved in the analgesic effect of AS1069562. In the study, AS1069562 improved allodynia in a mouse model of spinal hypersensitivity induced by intrathecal prostaglandin F2α, but duloxetine did not. Interestingly, the analgesic effect of AS1069562 on allodynia was blocked by intrathecal administration of the 5-HT1A receptor antagonist (S)-WAY100135.

### Table 3

<table>
<thead>
<tr>
<th>Drug</th>
<th>Locomotor Activity</th>
<th>Motor Coordination (Time on Rod)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>count</td>
<td>s</td>
</tr>
<tr>
<td>Vehicle</td>
<td>2544 ± 350</td>
<td>125 ± 6.6</td>
</tr>
<tr>
<td>AS1069562</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>2759 ± 338</td>
<td>108 ± 11</td>
</tr>
<tr>
<td>30 mg/kg</td>
<td>2695 ± 244</td>
<td>102 ± 8.1</td>
</tr>
<tr>
<td>100 mg/kg</td>
<td>2332 ± 351</td>
<td>112 ± 11</td>
</tr>
<tr>
<td>Vehicle</td>
<td>2915 ± 278</td>
<td>137 ± 16</td>
</tr>
<tr>
<td>Duloxetine</td>
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<td></td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>2731 ± 407</td>
<td>123 ± 8.9</td>
</tr>
<tr>
<td>30 mg/kg</td>
<td>1985 ± 322</td>
<td>105 ± 11</td>
</tr>
<tr>
<td>100 mg/kg</td>
<td>1451 ± 135**</td>
<td>133 ± 15</td>
</tr>
<tr>
<td>Vehicle</td>
<td>2560 ± 299</td>
<td>117 ± 14</td>
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<tr>
<td>Amitriptyline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 mg/kg</td>
<td>2221 ± 194</td>
<td>127 ± 13</td>
</tr>
<tr>
<td>100 mg/kg</td>
<td>1863 ± 188</td>
<td>107 ± 6.9</td>
</tr>
<tr>
<td>300 mg/kg</td>
<td>1276 ± 195**</td>
<td>113 ± 6.7</td>
</tr>
</tbody>
</table>

*P < 0.05; **P < 0.01; ***P < 0.001 by Dunnett’s test vs. vehicle-treated group.

![Fig. 8. Effects of AS1069562 (A), duloxetine (B), and amitriptyline (C) on gastric emptying ability in rats. AS1069562, duloxetine, and amitriptyline were administered orally 15 minutes before collecting stomachs. Each bar represents the mean ± S.E.M. for six rats. *P < 0.05; **P < 0.01; ***P < 0.001 by Dunnett’s test vs. vehicle-treated group.](image-url)
(4-(2-methoxyphenyl)-piperazin-1-yl)-2-phenylpropanamide] and 5-HT receptor agonist SR57227 [1-(6-chloro-2-pyridinyl)-4-piperidinamine], suggesting that AS1069562 has 5-HT \textsubscript{1A} agonist and 5-HT \textsubscript{3} antagonist activities in vivo (Murai et al., submitted for publication). Activation of spinal 5-HT \textsubscript{1A} receptor and inhibition of spinal 5-HT \textsubscript{3} receptor has been reported to produce analgesic effects. For example, the 5-HT \textsubscript{1A} receptor agonist F13640 markedly reduces analgesia in arthritic rats and decreases allodynic responses to tactile and thermal stimulation in rats sustaining spinal cord or sciatic nerve injury (Colpaert et al., 2002); intrathecally administered 5-HT \textsubscript{3} receptor antagonists reduced the second phase of formalin and decreases allodynic responses to tactile and thermal stimulation in rats sustaining spinal cord or sciatic nerve injury (Colpaert et al., 2002); and the 5-HT \textsubscript{3} antagonist ondansetron inhibited thermally and mechanically evoked neuronal responses of wide-dynamic-range neurons of rats 14 days after spinal nerve ligation (Suzuki et al., 2004). Although further investigation is needed, other mechanisms such as an agonistic action on 5-HT \textsubscript{1A} receptors and an antagonistic action on 5-HT \textsubscript{3} receptors could contribute to the analgesic effects of AS1069562, with its different profile from currently used antidepressants.

We also demonstrated that AS1069562 significantly reduced gastric emptying ability only at an oral dose of 30 mg/kg, while exerting analgesia of mechanical allodynia, thermal hyperalgesia, and spontaneous pain behavior in CCI rats. In contrast, duloxetine and amitriptyline significantly reduced gastric emptying at lower doses than those exerting analgesic effects. Furthermore, AS1069562 had no significant effect on locomotor activity or motor coordination at doses up to 100 mg/kg. In contrast, duloxetine and amitriptyline significantly reduced locomotor activity at 100 and 300 mg/kg, respectively. Based on the median efficacious dose of 30 mg/kg in three pain assays and the significant effect on gastric emptying at 30 mg/kg, the therapeutic index for AS1069562 was determined to be 1. In contrast, based on the median efficacious dose for duloxetine and amitriptyline (30 and 100 mg/kg, respectively) in three pain assays and the significant effect on gastric emptying (3 and 10 mg/kg, respectively), the therapeutic index for both duloxetine and amitriptyline was 0.1. In patients with neuropathic pain, gastrointestinal side effects such as nausea and vomiting are frequently caused by duloxetine and amitriptyline and are problematic because such adverse events limit the ability of clinicians to escalate doses to obtain efficacy and can cause early discontinuation of medication (Bystaier et al., 2005; Rosenzweig-Lipson et al., 2007). Although therapeutic indexes of AS1069562 and currently used antidepressants differed in this animal study, whether this difference is clinically meaningful in humans remains unclear. The reason why AS1069562 has a slightly preferable therapeutic index compared with current antidepressants in this study remains unclear; however, peripheral 5-HT may mediate nausea by activating 5-HT \textsubscript{3} receptors in the gastrointestinal tract on visceral afferent fibers to the vomiting center (Bergeron and Blier, 1994; Bailey et al., 1995). In the present study, AS1069562 inhibited 5-HT reuptake activity with lower potency than currently available antidepressants, suggesting that AS1069562 may have a smaller effect on peripheral 5-HT level than current antidepressants. Therefore, we speculate that the difference between AS1069562 and currently available antidepressants in the potency of 5-HT reuptake inhibition, which may influence the peripheral 5-HT level, might have contributed to the difference in the doses that cause nausea. However, further investigation of the effects of AS1069562 and currently available antidepressants on peripheral 5-HT level is needed. In addition, AS1069562 has an affinity for 5-HT \textsubscript{3} receptors in binding assay at almost the same potency as 5-HT reuptake inhibition and also has 5-HT \textsubscript{3} antagonist activity in vivo (Murai et al., submitted for publication). AS1069562 may inhibit nausea via its antagonistic action on 5-HT \textsubscript{3} receptors in the gastrointestinal tract on visceral afferent fibers to the vomiting center at a similar dose as 5-HT transporter inhibition.

In summary, AS1069562, the (+)-isomer of indeloxatine, broadly improved various types of neuropathic pain–related behaviors in CCI rats with unique characteristics in spinal monoamine turnover. These findings suggest that AS1069562 has potential as a treatment option for patients with neuropathic pain, with a different profile from currently available antidepressants.

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Authorship Contributions

**Participated in research design:** Murai, Tamura, Aoki, Matsuoka.

**Conducted experiments:** Murai, Tamura, Sekizawa, Kakimoto, Tsukamoto, Oe, Enomoto, Hamakawa.

**Performed data analysis:** Murai, Sekizawa, Kakimoto, Tsukamoto, Oe, Enomoto, Hamakawa.

**Wrote or contributed to the writing of the manuscript:** Murai, Aoki.

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