AS1069562, the (+)-isomer of indeloxazine, exerts analgesic effects in a rat model of neuropathic pain with unique characteristics in spinal monoamine turnover

Nobuhito Murai, Toshiaki Aoki, Seiji Tamura, Toshihiro Sekizawa, Shuichiro Kakimoto, Mina Tsukamoto, Tomoya Oe, Ryugo Enomoto, Nozomu Hamakawa, Nobuya Matsuoka

Running title: Analgesic effect of AS1069562 in rat neuropathic pain model

Address correspondence to: Nobuhito Murai

Department of Neuroscience
Pharmacology Research Labs
Drug Discovery Research
Astellas Pharma Inc.
21 Miyukigaoka, Tsukuba, Ibaraki, 305-8585, Japan
Tel: +81-29-863-7174
Fax: +81-29-856-2515
E-mail: nobuhito.murai@astellas.com

Number of text pages: 44
Number of tables: 3
Number of figures: 8
Number of references: 30
Number of words in Abstract: 246
Number of words in Introduction: 503
Number of words in Discussion: 1397

Abbreviations: 5-HT, serotonin; NE, norepinephrine; DA, dopamine, 5-hydroxyindoleacetic acid; 5-HIAA, 4-hydroxy-3-methoxyphenylglycol; MHPG, 4-hydroxy-3-methoxyphenylglycol; SNRI, serotonin and norepinephrine reuptake inhibitor; ANOVA, analysis of variance; CI, confidence interval; CCI, chronic constriction injury; RTX, resiniferatoxin
Recommended section assignment: Neuropharmacology
Abstract

AS1069562 is the (+)-isomer of indeloxazine, which had been clinically used for the treatment of cerebrovascular diseases with multiple pharmacological actions including serotonin (5-HT) and norepinephrine (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 current antidepressants duloxetine and amitriptyline. AS1069562 significantly elevated extracellular 5-HT and NE levels in the rat spinal dorsal horn, though its 5-HT and NE reuptake inhibition was much weaker than that of duloxetine in vitro. In addition, AS1069562 increased the ratio of both 5-HT and NE to their metabolites contents in the rat spinal cord, while duloxetine slightly increased only the ratio of 5-HT to its metabolite contents. 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. Further, AS1069562, duloxetine and amitriptyline significantly improved spontaneous pain-associated behavior. In a gastric emptying study, AS1069562 affected gastric emptying at the same dose exerting 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 current 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 serotonine (5-HT) and norepinephrine (NE) reuptake inhibitor (SNRI) 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, while 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 result in insufficient dose escalation for obtaining enough efficacy and an early discontinuation of the medication (Bymaster et al., 2005; Rosenzweig-Lipson et al., 2007). As such, more efficacious and safer agents than presently available compounds with different profiles from current antidepressants are urgently needed.

Indeloxazine is considered to be a cerebral metabolic enhancer with cerebral activating property, that is to say, indeloxatine shows facilitatory effects on learning behavior and protective effects against cerebral injuries by increasing in ATP and glucose contents 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 post-stroke depression, emotional disturbance, and reduced volition (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 the rat prefrontal cortex (Yamamoto et al., 1993), increase in extracellular levels of acetylcholine in the 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 contents in the 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 the rat brain, facilitatory effect on spontaneous electroencephalogram (EEG) in rats, and 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 current antidepressants duloxetine and amitriptyline.
Materials and Methods

Materials

(R)-2-[(1H-inden-7-yloxy)methyl]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 resiniferatoxin (RTX) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 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% Tween80, and 80% saline and was administered subcutaneously at an administration volume of 2 ml/kg. Drug concentrations were calculated in terms of free base. AS1069562 and amitriptyline were orally administered 1 h before behavioral testing or sampling, duloxetine was orally administered 3 h before behavioral testing or sampling, and RTX was subcutaneously administered 48 h 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-h light-dark cycle (light on: 7:30-19:30) 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, USA) 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. Lipofectamine2000 transfection regent (Invitrogen, Carlsbad, MO, USA) was used to create stable transfections and 800 µg/ml G-418 was used as a selection agent. Positive clones which 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 and 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 pargyline, pH7.3) containing 0.1% bovine serum albumin and samples were then pre-incubated at 25 °C for 10 min with KRH buffer containing
various concentrations of drugs. Subsequently, the cells were incubated for 20 min at 25 °C with KRH buffer containing approximately 25 nM \([^{3}\text{H}]\)-5-HT, \([^{3}\text{H}]\)-NE or \([^{3}\text{H}]\)-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 Microscinti-PS (ParkinElmer, Boston, MA, USA) 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) (ParkinElmer). For 5-HT transporter binding assay, \([\text{Benzene ring-}^{3}\text{H(N)}]\)-imipramine hydrochloride (1.4 nM, ParkinElmer) and competitor (imipramine hydrochloride, Sigma-Aldrich) were incubated at 25 °C for 30 min in 50 mM Tris-HCl (pH 7.4) buffer containing 100 mM NaCl. For NE transporter binding assay, \([\text{N-methyl}^{3}\text{H}]\)-nisoxetine hydrochloride (0.45 nM, ParkinElmer) and competitor (desipramine hydrochloride, Sigma-Aldrich) were incubated at 4 °C for 60 min in 50 mM Tris-HCl (pH 7.4) buffer containing 120 mM NaCl and 5 mM KCl. For DA transporter binding assay, \([\text{N-methyl}^{3}\text{H}]\)-WIN35,428 (1.7 nM, ParkinElmer) and competitor (GBR12909 dihydrochloride, Sigma-Aldrich) were incubated at 4 °C for 120 min 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 (ParkinElmer) was added, and the radioactivity of the filter paper was determined via scintillation counting. Specific binding was defined as a portion of total binding, which was replaced by 100 µM imipramine hydrochloride, desipramine hydrochloride, or GBR12909 dihydrochloride, for 5-HT, NE, or DA assay, respectively. The dissociation constant (K_d) and binding site density (B_max) were calculated via Scatchard analysis, while IC_{50} values were calculated via nonlinear regression analysis. The values of the apparent equilibrium dissociation constant of inhibitors (K_i) 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, USA) 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, USA) was passed through the fiber. One end of the fiber was attached to 2-cm polyethylene catheters (PE-10; Becton Dickinson, Franklin Lakes, NJ, USA), 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 vertebra 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 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 to 24 h 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 min. 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 (ECD) system (HTEC-500, Eicom, Kyoto, Japan).

**Monoamine and their metabolite contents in spinal cord**

Rat lumber spinal cord samples were collected and homogenized with 0.2 M perchloric
acid and 100 μM EDTA. After centrifugation (1500 g, 10 min, 4 °C), the supernatant was separated and stored at –80 °C until analysis. Five rats were used in each group. The concentrations of monoamine (5-HT and NE) and their metabolites (5-hydroxyindoleacetic acid (5-HIAA) and 4-hydroxy-3-methoxyphenylglycol (MHPG), respectively) in the samples were quantified using HPLC-ECD system (ECD-300, Eicom, Kyoto, Japan).

**Pharmacokinetics study**

Plasma samples were collected from rats at 0.25, 0.5, 1, 4, and 8 h after oral administration of 10 mg/kg AS1069562 or 0.5, 1, 2, 3, 4, and 8 h after oral administration of 10 mg/kg duloxetine. Brain samples were collected from rats at 1 h after oral administration of 10 mg/kg AS1069562 or 3 h 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 Na-K phosphate buffer (pH 7.4). Plasma and brain samples were then subjected to protein precipitation with acetonitrile. After centrifugation (1500 g, 10 min, 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, USA).

**Chronic constriction injury (CCI) surgery**
CCI surgery was performed in accordance with the method of Bennett and Xie (Bennett and Xie, 1988). Animals were anesthetized with pentobarbital, and the left common sciatic nerve was exposed at the level of the mid-thigh 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 approximately 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 (von Frey hair test)**

The cutaneous nociceptive threshold was measured by modification of a previously reported method (Chaplan et al., 1994). Briefly, 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 s, 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 higher 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). Briefly, 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 cut-off point was set at 15 s to prevent tissue damage. The apparatus was calibrated to give a paw withdrawal latency of approximately 10 s in naïve 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 (SCT-MAG-TF; Neuroscience Inc. Tokyo, Japan; 1 mm in diameter, 3 mm long) was implanted into the subcutaneous space of the left hind limb dorsum under isoflurane anaesthesia. Two or more days after the implantation, the animals were acclimated to dim light (approximately 20 lux) for at least 20 min and then placed inside the test chamber (NS-SCT10R, Neuroscience Inc.; 28 cm in diameter, 25 cm in height), which was surrounded by a round coil of 7000 turns. The chamber was set to dim light and constant room temperature (23 ± 2 °C). Measurements began after a 5-min 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® analysing software (NS-SC-S100; Neuroscience Inc.). Analysis parameters for the limb movement related waveforms were set at a frequency range of 2.5 to 20 Hz, with a threshold of 0.01 V, minimum duration of 0.09 sec and minimum gap duration of 0.03 s. CCI rats exhibit frequent aberrant asymmetrical 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 min 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 assessing, and 1.5% methylcellulose 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 min 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 2000 g for 10 min at 4 °C. The supernatant was separated via high-speed centrifugation at 20000 g for 10 min 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, USA). 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 min for 60 min. 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 min. Each rat was subjected to three training sessions. Rats that remained on the rod for over 90 s 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.

Statistical Analysis

Data were expressed as the mean and 95% confidence interval for uptake assay, as the mean ± standard deviation (S.D.) for pharmacokinetic profiles, and as the mean ± standard error of means (S.E.M.) for binding affinity, extracellular monoamine levels, monoamine and their metabolite contents, CCI model, gastric emptying ability, locomotor activity, and rotarod performance assessment. 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 comparison tests. P <0.05 was considered significant.
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\textsubscript{50} values of 0.35 and 3.3 μM in HEK293 cells expressing recombinant human transporters, and of 0.38 and 4.0 μM in HEK293 cells expressing recombinant rat transporters, respectively. The potencies of AS1069562 for inhibiting these reuptakes were lower (approximately 9-fold for 5-HT and approximately 310-fold for NE in rats) than that of duloxetine. AS1069562 did not inhibit DA reuptake activities. The results are summarized in Table 1.

Binding affinity of AS1069562 to 5-HT, NE, and DA transporters.

AS1069562 had affinities to human 5-HT, NE, and DA transporters with respective K\textsubscript{i} values of 0.040 ± 0.0032 μM, 4.4 ± 0.15 μM, and 4.8 ± 0.21 μM.

Effects of AS1069562 and duloxetine on extracellular monoamine levels in rat spinal dorsal horns

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 approximately 192% of that of duloxetine and elevated extracellular NE levels to approximately 43% of that of duloxetine (Fig. 2).
Effects of AS1069562 and duloxetine on monoamines and their metabolite contents in rat spinal cords

While AS1069562 orally administered at 10 and 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). Further, AS1069562 orally administered at 10 and 30 mg/kg significantly and dramatically increased the ratios of both 5-HT/5-HIAA and NE/MHPG. In contrast, 30 mg/kg duloxetine significantly but slightly increased only the ratio of 5-HT/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 h after oral administration of 10 mg/kg in rats (Fig. 4). The brain levels of AS1069562 at 1 h 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 15 ng/ml (45 nM) 3 h after oral administration of 10 mg/kg in rats (Fig. 4). The brain levels of duloxetine at 3 h 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 100 mg/kg with an MPE of 155% (Fig. 6C). Although duloxetine did not show significant amelioration, ameliorating trend was noted at 30 mg/kg with an MPE of 61% (Fig. 6B).

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 a subcutaneous 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 an 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 (10 and 30 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 48% at doses over 3 mg/kg and by 22% 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 the locomotor activity at 100 mg/kg 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 mg/kg, 100 mg/kg, and 300 mg/kg, respectively (Table 3).
Discussion

Here, we demonstrated that AS1069562 inhibited 5-HT and NE reuptake activities in the 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 approximately 192% of that of duloxetine, and extracellular NE levels to approximately 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 5-HIAA and MHPG contents, 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 to 5-HT_{1B} receptor at almost the same potency as 5-HT reuptake inhibition (paper submitted for publication). Given that 5-HT_{1B} receptors have
been reported to act as autoreceptors, inhibiting the release of 5-HT at serotonergic nerve terminals (De Groote et al., 2003; Pytliak et al., 2011), AS1069562 may enhance release of 5-HT via its effect on 5-HT<sub>1B</sub> 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 to 5-HT<sub>1B</sub> receptors, with a Ki value of 4.0 µM which was approximately 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, 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 only suppressed CCI-induced mechanical allodynia 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 over 10 mg/kg. Although AS1069562 inhibited rat 5-HT reuptake activities by approximately 9-fold and NE reuptake activities by approximately 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 why 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 to 5-HT1A and 5-HT3 receptors at almost the same potency as 5-HT reuptake inhibition, 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 (PGF2α), 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 and 5-HT3 receptor agonist SR57227, suggesting that AS1069562 has a 5-HT1A agonist and
5-HT₃ antagonist activities in vivo (paper submitted for publication). Activation of spinal 5-HT₁₄ receptor and inhibition of spinal 5-HT₃ receptor has been reported to produce analgesic effects. For example, 5-HT₁₄ 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-HT₃ receptor antagonists reduced the second phase of formalin-induced nociceptive behavior (Okamoto et al., 2004) and the 5-HT₃ 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 of 5-HT₁₄ receptor and an antagonistic action of 5-HT₃ receptor could contribute to the analgesic effects of AS1069562 with different profile from current 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. Further, AS1069562 had no significant effect on locomotor activity or motor condition 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 3 pain assays and the significant effect on gastric emptying at 30 mg/kg, the therapeutic index (TI) 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 3 pain assays and the significant effect on gastric emptying (3 and 10 mg/kg, respectively), the TI 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 (Bymaster et al., 2005; Rosenzweig-Lipson et al., 2007). Although TIs of AS1069562 and currently used antidepressants differed in this animal study, whether or not this difference is clinically meaningful in humans remains unclear. The reason why AS1069562 has a slightly preferable TI compared with current antidepressants in this study remains unclear; however, peripheral 5-HT may mediate nausea by activating 5-HT$_3$ 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 current 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 current 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 which cause nausea. However, further investigation of the effects of AS1069562 and current antidepressants on peripheral 5-HT level is needed. In addition, AS1069562 has an affinity to 5-HT$_3$ receptors in binding
assay at almost the same potency as 5-HT reuptake inhibition and also has a 5-HT₃ antagonist activity *in vivo* (paper submitted for publication). AS1069562 may inhibit nausea via its antagonistic action of 5-HT₃ 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 current antidepressants.
Acknowledgements

The authors would like to acknowledge Tomonari Watabiki, Masanobu Iino, Ritsuko Matsuda, and Yuko Takahashi for their expert technical assistance.
Authorship Contributions

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

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

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

Wrote or contributed to the writing of the manuscript: Murai and Aoki.
References


Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K₁) and the concentration of inhibitor which causes 50 per cent inhibition (I₅₀) of an enzymatic reaction. *Biochem Pharmacol* 22:3099–3108.


Yamamoto M, Ooyama M, Ozawa Y, Okada M, Tada S, Yamaguchi T, and Endo H
(1993) Effects of indeloxazine hydrochloride, a cerebral activator, on passive avoidance learning impaired by disruption of cholinergic transmission in rats.

*Neuropharmacology* **32**:695-701.
Footnotes

All authors are employees of Astellas Pharma Inc. and have not received financial support from any other institution.

Please send reprint requests to: Mr. Nobuhito Murai

21 Miyukigaoka

Tsukuba, Ibaraki, 305-8585, Japan

E-mail: nobuhito.murai@astellas.com
Figures Legends

Figure 1. Chemical structure of AS1069562.

Figure 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 h, respectively, before sampling. Each bar represents the mean ± S.E.M. for 5 rats. *, \( p < 0.05 \); and ***, \( p < 0.001 \) by Dunnett’s test compared with the vehicle-treated group.

Figure 3. Effects of AS1069562 and duloxetine on the ratio of 5-HT to 5-HIAA (A) and NE to MHPG (B) contents in rat spinal cords. AS1069562 and duloxetine were orally administered 1 or 3 h, respectively, before collecting the spinal cords. Each bar represents the mean ± S.E.M. for 5 rats. **, \( p < 0.01 \); and ***, \( p < 0.001 \) by Dunnett’s test compared with the vehicle-treated group.

Figure 4. Pharmacokinetic profiles of AS1069562 and duloxetine in rats. Plasma concentrations (ng/ml) were measured 0.25, 0.5, 1, 4, and 8 h after oral administration of 10 mg/kg AS1069562 or 0.5, 1, 2, 3, 4, and 8 h after oral administration of 10 mg/kg duloxetine. Data are presented as the mean ± S.D. for 3 rats.

Figure 5. Analgesic effects of AS1069562 (A), duloxetine (B), amitriptyline (C), and RTX (D) on mechanical allodynia in CCI rats. AS1069562, duloxetine, and
amitriptyline were orally administered 1, 3, and 1 h, respectively, before the von Frey test and RTX was subcutaneously administered 48 h before. Each bar represents the mean ± S.E.M. for 8 to 11 rats. ###, \( p < 0.001 \) by Student’s \( t \) test compared with the control group. *, \( p < 0.05 \); and ***, \( p < 0.001 \) by Dunnett’s test compared with the vehicle-treated group. ††, \( p < 0.01 \) by Student’s \( t \) test compared with the vehicle-treated group.

**Figure 6.** Analgesic effects of AS1069562 (A), duloxetine (B), amitriptyline (C), and RTX (D) on thermal hyperalgesia in CCI rats. AS1069562 and amitriptyline were orally administered 1 h before the plantar test, duloxetine was orally administered 3 h before testing, and RTX was subcutaneously administered 48 h before testing. Each bar represents the mean ± S.E.M. for 8 to 11 rats. #, \( p < 0.05 \); ##, \( p < 0.01 \); and ###, \( p < 0.001 \) by Student’s \( t \) test compared with the control group. **, \( p < 0.01 \); and ###, \( p < 0.001 \) by Dunnett’s test compared with the vehicle-treated group. †††, \( p < 0.001 \) by Student’s \( t \) test compared with the vehicle-treated group. N.S., not significant by Dunnett’s test compared with the vehicle-treated group.

**Figure 7.** Analgesic effects of AS1069562 (A), duloxetine (B), and amitriptyline (C) on spontaneous pain-associated limb movement frequency in CCI rats. AS1069562 and amitriptyline were orally administered 1 h and duloxetine 3 h before the measurement of limb movement frequency. Each bar represents the mean ± S.E.M. for 9 to 12 rats. ##, \( p < 0.01 \); and ###, \( p < 0.001 \) by Student’s \( t \) test compared with the control group. *,
$p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ by Dunnett’s test compared with the vehicle-treated group.

**Figure 8.** Effects of AS1069562 (A), duloxetine (B), and amitriptyline (C) on gastric emptying ability in rats. AS1069562, duloxetine, and amitriptyline were orally administered 15 min before collecting stomachs. Each bar represents the mean ± S.E.M. for 6 rats. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ by Dunnett’s test compared with the vehicle-treated group.
Table 1. IC\textsubscript{50} values of AS1069562 and duloxetine for 5-HT, NE, and DA reuptake activities

Values are the mean (95% confidence interval).

<table>
<thead>
<tr>
<th></th>
<th>AS1069562</th>
<th>Duloxetine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\mu M)</td>
<td></td>
</tr>
<tr>
<td>5-HT</td>
<td>0.35 (0.27-0.47)</td>
<td>0.013 (0.0059-0.028)</td>
</tr>
<tr>
<td>Human</td>
<td>NE</td>
<td>3.3 (1.4-7.9)</td>
</tr>
<tr>
<td></td>
<td>DA</td>
<td>&gt;100</td>
</tr>
<tr>
<td>5-HT</td>
<td>0.38 (0.18-0.78)</td>
<td>0.042 (0.022-0.080)</td>
</tr>
<tr>
<td>Rat</td>
<td>NE</td>
<td>4.0 (3.5-4.6)</td>
</tr>
<tr>
<td></td>
<td>DA</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>
Table 2. Monoamines and their metabolite contents in rat spinal cord

Values are the mean ± S.E.M. for 5 rats.

<table>
<thead>
<tr>
<th></th>
<th>vehicle</th>
<th>AS1069562</th>
<th>vehicle</th>
<th>Duloxetine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>3 mg/kg</td>
<td>10 mg/kg</td>
<td>30 mg/kg</td>
</tr>
<tr>
<td>5-HT (ng/g)</td>
<td>590 ± 61</td>
<td>639 ± 30</td>
<td>771 ± 41*</td>
<td>785 ± 40*</td>
</tr>
<tr>
<td>5-HIAA (ng/g)</td>
<td>285 ± 26</td>
<td>301 ± 12</td>
<td>233 ± 18</td>
<td>151 ± 6.8***</td>
</tr>
<tr>
<td>NE (ng/g)</td>
<td>293 ± 3.3</td>
<td>276 ± 15</td>
<td>359 ± 25*</td>
<td>369 ± 13*</td>
</tr>
<tr>
<td>MHPG (ng/g)</td>
<td>25.2 ± 3.1</td>
<td>24.8 ± 3.5</td>
<td>15.3 ± 1.4*</td>
<td>12.3 ± 1.1**</td>
</tr>
</tbody>
</table>

*p < 0.05; **p < 0.01; and ***p < 0.001 by Dunnett’s test compared with the vehicle-treated group.
Table 3. Effects on locomotor activity and motor condition in rats

Values are the mean ± S.E.M. for 8 rats.

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

**p < 0.01 by Dunnett’s test compared with the vehicle-treated group.
Fig. 1.
Fig. 2.

A

5-HT (% of pre)

vehicle  10  30  30
AS1069562 (mg/kg p.o.)

B

NE (% of pre)

vehicle  10  30  30
AS1069562 (mg/kg p.o.)

Duloxetine (mg/kg p.o.)
Fig. 3.

A

5-HT / 5-HIAA

**

***

vehicle 3 10 30

AS1069562 (mg/kg p.o.)

B

NE / MHPG

**

***

vehicle 3 10 30

AS1069562 (mg/kg p.o.)
Fig. 4.
Fig. 5.
Fig. 7.

**A**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Limb Movement Frequency (events in 30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>Vehicle</td>
<td>150</td>
</tr>
<tr>
<td>3 mg/kg AS1069562</td>
<td>250</td>
</tr>
<tr>
<td>10 mg/kg AS1069562</td>
<td>300</td>
</tr>
<tr>
<td>30 mg/kg AS1069562</td>
<td>350</td>
</tr>
</tbody>
</table>

Significance:
- *p < 0.05
- **p < 0.01
- ***p < 0.001

**B**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Limb Movement Frequency (events in 30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>Vehicle</td>
<td>150</td>
</tr>
<tr>
<td>3 mg/kg Duloxetine</td>
<td>200</td>
</tr>
<tr>
<td>10 mg/kg Duloxetine</td>
<td>250</td>
</tr>
<tr>
<td>30 mg/kg Duloxetine</td>
<td>300</td>
</tr>
</tbody>
</table>

Significance:
- **p < 0.01
- ***p < 0.001

**C**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Limb Movement Frequency (events in 30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>Vehicle</td>
<td>150</td>
</tr>
<tr>
<td>10 mg/kg Amitriptyline</td>
<td>200</td>
</tr>
<tr>
<td>30 mg/kg Amitriptyline</td>
<td>250</td>
</tr>
<tr>
<td>100 mg/kg Amitriptyline</td>
<td>300</td>
</tr>
</tbody>
</table>

Significance:
- **p < 0.01
- ***p < 0.001
Fig. 8.