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Title:

Binding characteristics and analgesic effects of mirogabalin, a novel ligand for the $\alpha_2\delta$ subunit of voltage-gated calcium channels

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Running title:

Mirogabalin, a novel $\alpha_2\delta$ ligand

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Abbreviations:

AUC: area under the curve

B_{max}: maximum binding capacity

C_{max}: maximum concentration

CNS: central nervous system

ED_{50}: 50% effective dose

IC_{50}: 50% inhibitory concentration

K_d: dissociation constant

K_{off}: dissociation rate constant

PK: pharmacokinetics

PSL: partial sciatic nerve ligation

STZ: streptozotocin

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Abstract

Mirogabalin ([(1*R*,5*S*,6*S*)-6-(aminomethyl)-3-ethylbicyclo[3.2.0]hept-3-en-6-yl]acetic acid), a novel ligand for the $\alpha_2\delta$ subunit of voltage-gated calcium channels, is being developed to treat pain associated with diabetic peripheral neuropathy and postherpetic neuralgia. In the present study, we investigated the *in vitro* binding characteristics and *in vivo* analgesic effects of mirogabalin, compared to those of pregabalin, a standard $\alpha_2\delta$ ligand. Mirogabalin showed potent and selective binding affinities for the $\alpha_2\delta$ subunits, while having no effects on 186 off-target proteins. Similar to pregabalin, mirogabalin did not show clear subtype selectivity ($\alpha_2\delta$-1 vs $\alpha_2\delta$-2) or species differences (human vs rat). However, in contrast to pregabalin, mirogabalin showed greater binding affinities for human $\alpha_2\delta$-1, human $\alpha_2\delta$-2, rat $\alpha_2\delta$-1, and rat $\alpha_2\delta$-2 subunits; further, it had a slower dissociation rate for the $\alpha_2\delta$-1 subunit than the $\alpha_2\delta$-2 subunit. Additionally, in experimental neuropathic pain models, partial sciatic nerve ligation rats and streptozotocin-induced diabetic rats, mirogabalin showed more potent and longer lasting analgesic effects. In safety pharmacological evaluations, mirogabalin and pregabalin inhibited rota-rod performance and locomotor activity in rats; however, the safety indices of mirogabalin were superior to those of pregabalin. In conclusion, mirogabalin shows potent and selective binding affinities for the human and rat $\alpha_2\delta$ subunits, and slower dissociation rates for the $\alpha_2\delta$-1 subunit than the $\alpha_2\delta$-2 subunit. It shows potent and long-lasting analgesic effects in rat models of neuropathic pain, and wider safety margins for side effects of the central nervous system. These properties of mirogabalin can be associated with its unique binding characteristics.

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Introduction

Gabapentinoids, such as pregabalin and gabapentin, are selective ligands for the $\alpha_2\delta$ subunit of voltage-gated calcium channels (Li et al., 2011; Alexander et al., 2015). The predominant mechanism of action of gabapentinoids is inhibiting neurotransmitter release at the presynaptic endings of neurons. The inhibition of neurotransmitter (e.g., glutamate, substance P, and calcitonin gene-related peptide) release attenuates neuronal hyperexcitability in the brain and spinal cord and contributes to various pharmacological effects such as analgesic, anticonvulsant, and anxiolytic activity (Fehrenbacher et al., 2003; Sills 2006; Dooley et al., 2007; Taylor et al., 2007; Stahl et al., 2013). In fact, pregabalin and gabapentin have been licensed and used in many countries for pain, epilepsy, and generalized anxiety disorders (Stahl et al., 2013). Several scientific associations and regulatory agencies recommend pregabalin and gabapentin as the first-line drugs for the treatment of neuropathic pain (Argoff et al., 2006; Finnerup and Jensen, 2007; Dworkin et al., 2007; Attal et al., 2010; Dworkin et al., 2013; Cohen et al., 2015). However, the clinical utility of pregabalin and gabapentin is limited by central nervous system (CNS) side effects such as dizziness and somnolence (Freeman et al., 2008; Ziegler, 2008; Goodman and Brett, 2017); thus, there is an unmet need for further improvement in this class of drugs. Behavioral, neurochemical, and electrophysiological studies using transgenic mice have shown that the $\alpha_2\delta$-1 subunit of voltage-gated calcium channels contributes to analgesic effects (Lie et al., 2006; Field et al., 2006), whereas the $\alpha_2\delta$-2 subunit contributes to CNS side effects (Barclay et al., 2001; Brill et al., 2004), suggesting that ligand selectivity for $\alpha_2\delta$-1 and $\alpha_2\delta$-2, might result in different clinical outcomes.

Mirogabalin ([(1*R*,5*S*,6*S*)-6-(aminomethyl)-3-ethylbicyclo[3.2.0]hept-3-en-6-yl]acetic acid) is a novel ligand for the $\alpha_2\delta$ subunit of voltage-gated calcium channels that is being developed to treat pain associated with diabetic peripheral neuropathy and postherpetic neuralgia.

In the present study, we investigated the *in vitro* binding characteristics and *in vivo* analgesic effects of mirogabalin, compared to those of pregabalin.

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Materials and Methods

Chemicals

Mirogabalin besylate (code number: DS-5565), mirogabalin (free-form of DS-5565), and pregabalin were synthesized by Daiichi Sankyo Co., Ltd. (Tokyo, Japan). ^{3}H-mirogabalin (specific radioactivity: 756 GBq/mmol) and ^{3}H-pregabalin (specific radioactivity: 1280 GBq/mmol) were obtained from Sekisui Medical Co., Ltd. (Tokyo, Japan). The mirogabalin besylate and pregabalin were dissolved in distilled water. In the rota-rod and locomotor tests, mirogabalin besylate was suspended in a 0.5% methylcellulose solution because of its solubility limit. For an *in vitro* off-target profiling assay, mirogabalin besylate was dissolved in dimethyl sulfoxide. The dose levels of test compounds are expressed as free form. All other reagents were of analytical grade and obtained from conventional commercial sources. Chemical structures of mirogabalin besylate and pregabalin are shown in Fig. 1.

Cell membranes

The cell membrane fraction containing each $\alpha_2\delta$ subunit was prepared from the 293A stable cell line expressing each $\alpha_2\delta$ subunit as described in a previous report (Gee et al., 1996). Briefly, 293A cells (Thermo Fisher Scientific Inc., Waltham, MA) were co-transfected with 5 μg of $\alpha_2\delta$ subunit expression plasmid and 0.5 μg of pPURO (Clontech Laboratories, Inc., Mountain View, CA) by lipofection. The transfected cells were diluted and spread to 150 mm dishes. After 10 days culture with Dulbecco's modified Eagle medium (Thermo Fisher Scientific) containing 10% fetal bovine serum and 1 μg/ml puromycin (Thermo Fisher Scientific), cell colonies were picked up and selected by expression level of $\alpha_2\delta$ subunit examined by Western blotting assay. For Western blotting assay, anti-$\alpha_2\delta$-1 antibody (C5105, Sigma-Aldrich, St. Louis, MO) and anti-$\alpha_2\delta$-2 antibody (sc-34768, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used for detection of $\alpha_2\delta$-1 and $\alpha_2\delta$-2, respectively. The cells harvested by scraper were lysed by sonication on ice and the membrane fraction was collected by centrifugation. The pellet of membrane was washed with

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binding assay buffer (0.01 M HEPES pH 7.5, 0.1 M NaCl) three times by repetition of suspension and centrifugation. Total protein concentration of membrane fractions were determined by BCA protein assay kit (Thermo Fisher Scientific). The total protein concentrations of human $\alpha_2\delta$-1 subunit-expressing 293A cell membrane, human $\alpha_2\delta$-2 subunit-expressing 293A cell membrane, rat $\alpha_2\delta$-1 subunit-expressing 293A cell membrane and rat $\alpha_2\delta$-2 subunit-expressing 293A cell membrane were 11.2 mg/ml, 10.7 mg/ml, 9.8 mg/ml and 5.7 mg/ml, respectively.

Animals

Male Crl:CD (SD) rats and F344/DuCrlCrlj rats (Charles River Laboratories Japan, Inc., Kanagawa, Japan), as well as BN/SsN Slc rats (Japan SLC, Inc., Shizuoka, Japan) were used. The animals were housed under regulated conditions for temperature (19–26°C), relative humidity (35–75%) and a 12 h light-dark cycle (lights on 7:00–19:00 h), with a commercial diet and tap water available *ad libitum*. Animals were randomly allocated to the study groups by computed randomization procedure based on paw withdrawal threshold (neuropathic pain models) or random number generation (safety pharmacological evaluations). All procedures for pain assessment were conducted by an experimenter blinded to the treatment conditions.

All experimental procedures were performed in accordance with the Basic Guidelines for the Use of Experimental Animals in Institutions under the Jurisdiction of the Ministry of Health, Labour and Welfare (Notification No. 0601001 of the Science Bureau, Japanese Ministry of Health, Labour and Welfare, June 1, 2006), the Guidelines for Animal Studies (Nonclinical Research Center and Toxicological Science Division, Mitsubishi Chemical Medience Corporation) and the Guideline of the Institutional Animal Care and Use Committee of Daiichi Sankyo Co., Ltd.

In vitro binding profile for the $\alpha_2\delta$ subunits

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Saturation assay

The cell membranes prepared as described above were diluted with binding assay buffer (0.01 M HEPES pH 7.5, 0.1 M NaCl) on ice. The diluted cell membranes (final concentration of 0.1 mg protein/ml) and ^{3}H-labeled compound serial dilutions (final concentration of 0.39–100 nM ^{3}H-mirogabalin or 0.78–200 nM ^{3}H-pregabalin) were mixed in each well of a 96U plate (MS-3296U, Sumitomo Bakelite Co., Ltd., Tokyo, Japan), and incubated for 4 h at room temperature (N = 4). After incubation, the membranes from each well were collected in a 96-well UNIFILTER (GF/B Filter 7700-3303, Whatman Inc., Clifton, NJ) using vacuum manifold. Each filter was washed 3 times with 400 μl of binding assay buffer and dried overnight at room temperature. Radioactivity of the dried filter picked up from each well was counted by the liquid scintillation counter (PerkinElmer Inc., Waltham, MA) after immersed in 4 ml of Pico-Fluor 40 (PerkinElmer) in a glass vial. Non-specific binding was defined as the residual binding in the presence of 100 μM of mirogabalin or 200 μM of pregabalin.

Dissociation kinetic assay

The cell membranes (final concentration of 0.1 mg protein/ml) and ^{3}H-labeled compound solutions (final concentration of 10 nM ^{3}H-mirogabalin or 20 nM ^{3}H-pregabalin) were incubated for 4 h at room temperature as described above. After that, dissociation reaction was initiated by adding 2 μl of the corresponding unlabeled compound solution (10 mM mirogabalin or 20 mM pregabalin) and the reaction mixture was incubated for 0 (immediately), 0.5, 1, 2, 4, 6 and 10 h at room temperature (N = 4). After incubation, the membrane-bound radioligand was recovered by filtration and the radioactivity was determined as described above. Non-specific binding was defined as the residual binding after 10 h of incubation in the presence of 200 μM of mirogabalin or 400 of μM pregabalin.

In vitro off-target pharmacological profile

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The *in vitro* pharmacological activity of mirogabalin besylate on a total of 187 receptors, channels, transporters and enzymes was evaluated by radioligand binding and enzyme assays, using the standard protocols provided by Eurofins Panlabs Taiwan Ltd. (formerly MDS Pharma Services Taiwan Ltd., Taipei, Taiwan). The protocols consisted of the SpectrumScreen® package, 7 additional binding assays and 14 enzyme assays. The primary assays were performed at a concentration of 50 μM in duplicate, and when significant responses (≥ 50% inhibition) were noted, the 50% inhibitory concentration (IC_{50}) values were determined in the follow-up assays.

Partial sciatic nerve ligation model in rats

Eighty-four male Sprague Dawley rats were divided into groups of 12. The partial sciatic nerve ligation (PSL) model was prepared according to a previously used method (Seltzer et al., 1990). Under 2% isoflurane anesthesia (Escain®, Mylan Inc., Osaka, Japan), the left femoral skin was incised and the sciatic nerve was exposed. Approximately 1/2 to 1/3 of the sciatic nerve was tightly ligated with surgical 8-0 silk thread. The surgical area was sterilized with kanamycin (Meiji Seika Pharma Co., Ltd., Tokyo, Japan) and was sutured with surgical 4-0 silk thread. Twelve days after PSL, the paw withdrawal threshold was measured using the Dynamic Plantar Aesthesiometer (37400, Ugo Basile, Varese, Italy). Mechanical stimulation was applied to the left hind paw in a stepwise manner (from 0 to 30 g in 40 s, at 0.5 g/step). PSL rats with the paw withdrawal threshold of 7 g or less were selected and assigned to treatment groups. The rats received the test compound or vehicle (control) orally and the paw withdrawal threshold was measured at 0 (before administration), 2, 4, 6 and 8 h after administration. The area under the curve of the paw withdrawal threshold (paw withdrawal threshold $AUC_{0–8h}$) was calculated by the trapezoidal method.

Streptozotocin-induced diabetic model in rats

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Seventy diabetic male Brown Norway rats were divided into groups of 10. Ten normal male rats were used as the non-diabetic control. Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ; 60 mg/kg in 0.1 M citrate buffer at pH 4.5), and the rats with glucose levels > 300 mg/dL were defined as diabetic, 7 days after the STZ injection (diabetic successful rate: 59%). Sixteen weeks after the STZ injection, the paw withdrawal threshold to mechanical stimulation was determined using von Frey filaments (North Coast Medical Inc., Gilroy, CA). STZ rats with the paw withdrawal threshold of 6 g or less were selected and assigned to treatment groups. The rats received the test compound or vehicle (control) orally for 5 days (twice a day from Day 1 to Day 4, and once a day on Day 5). The von Frey test was performed at 0 (before administration), 2, 4, 6, 8 and 12 h after the first administration on Day 1, Day 3 and Day 5. The area under the curve of the paw withdrawal threshold (paw withdrawal threshold $AUC_{0–12h}$) was calculated by the trapezoidal method. Satellite groups (3 animals/group) were set aside for a pharmacokinetics (PK) evaluation. Blood samples were collected from the tail vein using heparinized micro-hematocrit capillary tubes and the plasma concentrations of mirogabalin and pregabalin were determined by the validated liquid chromatography–mass spectrometry/mass spectrometry method.

Rota-rod performance in rats

Ninety-six male Fischer rats were divided into groups of 8. Rats able to walk on the rota-rod (6 rpm, 9 cm in diameter, KN-75, Natsume Seisakusho Co., Ltd., Tokyo, Japan) for 3 min were selected. After oral administration of the test compound or vehicle (control), each rat was placed on the rota-rod, and motor coordination was considered to be impaired if the rat fell off the rota-rod within 60 s in 3 trials. The rota-rod test was conducted at 0 (before administration), 2, 4, 6, 8 and 24 h after administration.

Spontaneous locomotor activity in rats

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Eighty male Fischer rats were divided into groups of 8. After oral administration of the test compound or vehicle (control), locomotor activity was measured for 1 h using the Supermex system (SM-32, Muromachi Kikai Co., Ltd., Tokyo, Japan). Based on the time of peak effects of the test compounds in the rota-rod test, the pretreatment time was set at 6 h for mirogabalin besylate and 4 h for pregabalin.

Statistical analysis

For the *in vitro* saturation assay and dissociation kinetic assay, the maximum binding capacity (B_{max}), dissociation constant (K_d), dissociation rate constant (K_{off}) and dissociation half-life, including their 95% confidence limits, were calculated. For paw withdrawal threshold AUC and locomotor activity, the statistical analysis was performed by Dunnett's multiple comparison. Rota-rod performance was analyzed by Fisher's exact probability test. In addition, the Spearman's correlation coefficient was calculated to evaluate the dose-response relationship from the paw withdrawal threshold AUC. Differences were considered significant when $p < .05$. The 50% effective dose (ED_{50}) values and their 95% confidence limits were calculated by log-linear or logistic regression analysis and probit analysis. SAS Software Release 8.2 or 9.1.3 (SAS Institute Japan Ltd., Tokyo, Japan), EXSUS Version 7.7 (CAC Croit Corporation, Tokyo, Japan) and Microsoft Excel 2003 (Microsoft Japan Co., Ltd., Tokyo, Japan) were used for these analyses. Sample sizes were determined to provide > 80% power based on the results of preliminary studies and historical data.

Results

In vitro binding profile for the $\alpha_2\delta$ subunits

Saturation assay

Binding parameters of ^{3}H-mirogabalin and ^{3}H-pregabalin for the $\alpha_2\delta$ subunits are summarized in Table 1. The K_d values of mirogabalin for the human $\alpha_2\delta$-1, human $\alpha_2\delta$-2, rat $\alpha_2\delta$-1, and rat $\alpha_2\delta$-2 subunits were

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estimated to be 13.5, 22.7, 27.0, and 47.6 nM, respectively. The K_d values of pregabalin for the human $\alpha_2\delta$-1, human $\alpha_2\delta$-2, rat $\alpha_2\delta$-1, and rat $\alpha_2\delta$-2 subunits were estimated to be 62.5, 125.0, 142.9, and 166.7 nM, respectively. The B_{max} values of mirogabalin were similar to those of pregabalin. Therefore, mirogabalin showed greater binding affinities for the human and rat $\alpha_2\delta$ subunits, compared to pregabalin. Further, neither compound exhibited subtype selectivity ($\alpha_2\delta$-1 vs $\alpha_2\delta$-2) or species differences (human vs rat).

Dissociation kinetic assay

Dissociation kinetic parameters of ^{3}H-mirogabalin and ^{3}H-pregabalin for $\alpha_2\delta$ subunits are summarized in Table 2. Fig. 2 shows dissociation curves for the human $\alpha_2\delta$-1 and human $\alpha_2\delta$-2 subunits. The dissociation half-life of mirogabalin from the human $\alpha_2\delta$-1 and $\alpha_2\delta$-2 subunits were estimated to be 11.1 and 2.4 h, respectively. The dissociation half-life of pregabalin from the human $\alpha_2\delta$-1 and $\alpha_2\delta$-2 subunits were equivalent and estimated to be 1.4 h. The dissociation half-life of mirogabalin from the rat $\alpha_2\delta$-1 and $\alpha_2\delta$-2 subunits were estimated to be 8.7 and 2.3 h, respectively. The dissociation half-life of pregabalin from the rat $\alpha_2\delta$-1 and $\alpha_2\delta$-2 subunits were estimated to be 1.4 and 1.3 h, respectively. Therefore, mirogabalin had a longer dissociation half-life from the $\alpha_2\delta$-1 subunits than the $\alpha_2\delta$-2 subunits, in contrast to pregabalin.

In vitro off-target pharmacological profile

Mirogabalin showed binding affinity for the gabapentin binding site in rat cortical brain homogenates with the IC_{50} value of 16.0 nM. Mirogabalin had no effect on any other receptors, channels, transporters or enzymes at 50 μM.

Partial sciatic nerve ligation model in rats

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Fig. 3A shows the dose-response and time-course changes of paw withdrawal threshold in each treatment group; the results for the paw withdrawal threshold AUC are shown in Fig. 3B. Mirogabalin (3 and 10 mg/kg) and pregabalin (10 and 30 mg/kg) significantly increased paw withdrawal threshold $AUC_{0–8h}$ in a dose-dependent manner. The effect of mirogabalin peaked at 4 h after administration and remained there until 6 or 8 h after administration. The effect of pregabalin peaked at 4 h after administration and returned to the vehicle control level after 6 h.

Streptozotocin-induced diabetic model in rats

Fig. 4A shows the dose-response and time-course changes of paw withdrawal threshold in each treatment group; the results for the paw withdrawal threshold AUC are shown in Fig. 4B. The paw withdrawal threshold in vehicle control rats was lower than that in normal control rats. Mirogabalin (2.5, 5, and 10 mg/kg) and pregabalin (10, 20, and 40 mg/kg) significantly increased paw withdrawal threshold $AUC_{0–12h}$ in a dose-dependent manner. The effects of mirogabalin and pregabalin peaked at 4 h after administration, and there were no apparent differences in the maximum effects of both compounds. The analgesic effects of mirogabalin were enhanced by repeated dosing, while those of pregabalin showed no apparent changes. The ED_{50} values for mirogabalin on Day 1, Day 3 and Day 5 were estimated to be 4.4, 3.1 and < 2.5 mg/kg, respectively. The ED_{50} values for pregabalin on Day 1, Day 3 and Day 5 were estimated to be 26.8, 22.4 and 29.3 mg/kg, respectively.

Fig. 5 shows the dose-response and time-course changes in plasma concentrations of mirogabalin and pregabalin in each satellite group on Day 5, and the inset shows the dose-proportionality of C_{max} and $AUC_{0–12h}$. Table 3 summarizes the PK parameters on Day 1 and Day 5. The C_{max} and $AUC_{0–12h}$ of mirogabalin and pregabalin increased nearly dose-proportionally. There were no apparent changes in these parameters for mirogabalin and pregabalin, even after repeated dosing.

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Rota-rod performance in rats

Fig. 6 shows the dose-response and time-course changes of rota-rod performance in each treatment group. Mirogabalin had no significant effect on rota-rod performance at 1 and 3 mg/kg, and it significantly inhibited rota-rod performance at 10, 30 and 100 mg/kg. The maximum effect was observed 4–6 h after administration and the ED_{50} values were estimated to be 9.5 and 9.4 mg/kg at 4 and 6 h after administration, respectively. Pregabalin had no significant effect on rota-rod performance at 3 and 10 mg/kg, and it significantly inhibited rota-rod performance at 30, 100 and 300 mg/kg. The maximum effect was observed 4–6 h after administration and the ED_{50} value was considered 11.7 mg/kg. All animals recovered 24 h after administration of mirogabalin and pregabalin, at all dosage levels.

Spontaneous locomotor activity in rats

Fig. 7 shows the dose-response changes of locomotor activity in each treatment group. Mirogabalin had no effect on locomotor activity at 3 and 10 mg/kg, and it significantly decreased locomotor activity at 30 and 100 mg/kg. Pregabalin had no effect on locomotor activity at 10 and 30 mg/kg, and it significantly decreased locomotor activity at 100 and 300 mg/kg. The ED_{50} values of mirogabalin and pregabalin were estimated to be 43.9 and 111.8 mg/kg, respectively.

Safety indices for CNS side effects in rats

Table 4 summarizes the CNS side effect profile of mirogabalin and pregabalin. Safety indices were obtained by calculating the ratio between side effect ED_{50} values (rota-rod ED_{50} or locomotor ED_{50}) and analgesic ED_{50} values on Day 1 in the STZ study. In rota-rod performance, the safety indices were 2.1 for mirogabalin and 0.4 for pregabalin. The safety index 0.4 (under 1) represents a negative margin, indicating that pregabalin induced ataxia at lower doses than its analgesic doses. In locomotor activity, the safety indices were 10.0 for mirogabalin and 4.2 for pregabalin. Therefore, the safety indices of

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mirogabalin were superior to those of pregabalin in both tests.

Discussion

The $\alpha_2\delta$ subunits are multifunctional, and have been reported to affect calcium channel trafficking as well as biophysical properties of calcium channel currents (Dolphin 2012; Dolphin 2013). Increased expression of $\alpha_2\delta$-1 mRNA and $\alpha_2\delta$-1 protein has been observed in the dorsal root ganglion and the spinal cord dorsal horn of rat models for neuropathic pain (Luo et al., 2001; Newton et al., 2001; Wang et al., 2002; Li et al., 2004; Bauer et al., 2009; Bauer et al., 2010; Boroujerdi et al., 2011). In addition, knock-down of $\alpha_2\delta$-1 subunits by antisense has been reported to inhibit tactile allodynia in these rat models (Li et al., 2004; Boroujerdi et al., 2011). Transgenic mice overexpressing the $\alpha_2\delta$-1 subunit in neuronal tissues have been reported to exhibit behavioral hypersensitivity (tactile allodynia and thermal hyperalgesia) and electrophysiological hyperexcitability in the dorsal root ganglion and spinal dorsal horn neurons (Lie et al., 2006). The $\alpha_2\delta$-1 knock-out mice have been reported to show markedly reduced behavioral sensitivity to mechanical and cold stimuli. The knock-out mice also showed a delayed development of mechanical hypersensitivity after PSL and loss of analgesic effect of pregabalin (Patel et al., 2013). Knock-in mice expressing a mutant $\alpha_2\delta$-1 subunit that does not bind pregabalin or gabapentin have been reported to develop neuropathic pain that is insensitive to these drugs (Field et al., 2006). These findings indicate that $\alpha_2\delta$-1 subunit has an important role in neuropathic pain states and the analgesic effects of gabapentinoids are mediated through the $\alpha_2\delta$-1 subunit. In contrast, $\alpha_2\delta$-2 subunit is dominantly expressed in the cerebellar Purkinje cells, and mutant mice with a $\alpha_2\delta$-2 subunit deletion have been reported to show ataxia, paroxysmal dyskinesia and absence seizures (Barclay et al., 2001; Brodbeck et al., 2002; Brill et al., 2004; Ivanov et al., 2004; Donato et al., 2006). Furthermore, human epileptic encephalopathies associated with $\alpha_2\delta$-2 mutations have been reported (Edvardson et al., 2013; Pippucci et al., 2013). These distinct roles of $\alpha_2\delta$-1 and $\alpha_2\delta$-2 subunits suggest that ligand selectivity for $\alpha_2\delta$-1 and $\alpha_2\delta$-2 might bring about separate analgesic effects and CNS side effects.

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In the present study, mirogabalin specifically bound to $\alpha_2\delta$ subunits with high affinity at 2-digit nanomolar concentrations and had no effects on a total of 186 off-target proteins at 3 orders of higher concentration (50 μM). The binding affinities of mirogabalin for the human $\alpha_2\delta$-1, human $\alpha_2\delta$-2, rat $\alpha_2\delta$-1, and rat $\alpha_2\delta$-2 were greater than those of pregabalin, and neither compound showed subtype selectivity ($\alpha_2\delta$-1 vs $\alpha_2\delta$-2) or species differences (human vs rat). Interestingly, though the subtype selectivity of mirogabalin is not significant in K_d values, mirogabalin showed longer dissociation half-lives against $\alpha_2\delta$-1 subunit than $\alpha_2\delta$-2 subunit both in human and rat. On the other hand, pregabalin showed equal dissociation half-lives from $\alpha_2\delta$-1 subunit and $\alpha_2\delta$-2 subunit in both human and rat. The binding affinity of mirogabalin and pregabalin for $\alpha_2\delta$-3 or $\alpha_2\delta$-4 subunit was not evaluated in the present study. Gabapentin and pregabalin have been reported to show no binding affinity for those subtypes (Marais et al., 2001; Qin et al., 2002; Taylor et al., 2007). To date, drugs showing significant binding to $\alpha_2\delta$-3 or $\alpha_2\delta$-4 subunit have not yet been reported.

In typical experimental neuropathic pain models, such as PSL rats and STZ-induced diabetic rats, mirogabalin showed more potent and longer lasting analgesic effects than pregabalin. The greater binding affinity of mirogabalin for $\alpha_2\delta$-1 subunit is considered to contribute to its potent analgesic effects. Interestingly, unlike pregabalin, the analgesic effects of mirogabalin were enhanced by repeated administration in STZ-induced diabetic rats. In the mirogabalin treatment groups, the paw withdrawal threshold AUCs increased day-by-day without increases in AUCs of plasma drug concentrations. Specifically, at 10 mg/kg of mirogabalin, the paw withdrawal thresholds before administration on Day 3 and Day 5 were at the same level as those of normal controls and were higher than those on Day 1, regardless of almost undetectable plasma drug concentrations. The above phenomena and differences from pregabalin might be potentially explained by the sustained binding affinity of mirogabalin for $\alpha_2\delta$-1 subunit, rather than its PK parameters.

Including the IC_{50} and K_d values, the dissociation half-life for a target protein is suggested to be an important factor to determine duration of pharmacological effects and target selectivity *in vivo* (Copeland et al., 2006). In safety pharmacological evaluations, mirogabalin and pregabalin dose-dependently

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inhibited rota-rod performance and locomotor activity in rats. These effects are the class-effects of gabapentinoids and suggested to be related to CNS side effects observed in clinical practice. In both rota-rod and locomotor tests, the safety indices of mirogabalin were superior to those of pregabalin. As described above, several studies using transgenic mice have shown that the $\alpha_2\delta$-1 subunit plays an important role in the analgesic effects of gabapentinoids, whereas the $\alpha_2\delta$-2 subunit is related to CNS side effects. Mirogabalin had a longer dissociation half-life from the $\alpha_2\delta$-1 subunit than the $\alpha_2\delta$-2 subunit, in contrast to pregabalin. The unique binding characteristics of mirogabalin might contribute to the wider safety margin for CNS side effects, as well as the long-lasting analgesic effects. The findings in the present study support the favorable outcomes obtained in the phase II proof-of-concept study of mirogabalin as treatment for patients with diabetic peripheral neuropathy (Vinik et al., 2014; Merante et al., 2017). This phase II study demonstrated that mirogabalin was effective and well tolerated at 15, 20 and 30 mg/day, given either once or twice daily with or without titration.

In conclusions, mirogabalin shows potent and selective binding affinities for the human and rat $\alpha_2\delta$ subunits, and slower dissociation rate for the $\alpha_2\delta$-1 than the $\alpha_2\delta$-2 subunit. It shows potent and long-lasting analgesic effects in rat models for neuropathic pain and wider safety margin for CNS side effects. The unique binding characteristics of mirogabalin might contribute to its high analgesic efficacy and wide safety margin.

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Authorship Contributions

Domon and Arakawa contributed equally to the work presented here and should therefore be regarded as equivalent first authors.

Participated in research design: Arakawa, Matsuda, Yamamura, Kai, and Kitano.

Conducted experiments: Domon, Arakawa, Inoue, Matsuda, Takahashi, Yamamura, Kai, and Kitano.

Performed data analysis: Domon, Yamamura, and Kitano.

Wrote or contributed to the writing of the manuscript: Matsuda, Yamamura, Kai, and Kitano.

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Footnotes:

The authors have no conflicts of interest to declare.

Portions of this study were previously presented as follows:

Yokoyama T, Arakawa N, Domon Y, Matsuda, Inoue T, Kitano Y, Takahashi M, Yamamura N, and Kai K. Pharmacological, Pharmacokinetics and Safety Profiles of DS-5565, a Novel $\alpha_2\delta$ Ligand. The 21st World Congress of Neurology, September 21–26, 2013, Vienna, Austria.

Yokoyama T, Arakawa N, Domon Y, Matsuda, Inoue T, Kitano Y, Takahashi M, Yamamura N, and Kai K. Pharmacological, Pharmacokinetics and Safety Profiles of DS-5565, a Novel $\alpha_2\delta$ Ligand. The 66th Annual Meeting of the American Academy of Neurology, April 26–May 3, 2014, Philadelphia, PA.

Arakawa N, Yokoyama T, Domon Y, Matsuda, Inoue T, Kitano Y, Takahashi M, Yamamura N, and Kai K. Pharmacological, Pharmacokinetics and Safety Profiles of DS-5565, a Novel $\alpha_2\delta$ Ligand. The 24th Annual Meeting of the Diabetic Neuropathy Study Group, September 12–14, 2014, Sopron, Hungary.

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Figure legends

Fig. 1. Chemical structures of mirogabalin besylate (upper) and pregabalin (lower).

Fig. 2. Dissociation curves of mirogabalin and pregabalin for the human $\alpha_2\delta$-1 and human $\alpha_2\delta$-2 subunits. Each value represents the mean ± S.E.M. (N = 4). Mirogabalin had a longer dissociation half-life from the $\alpha_2\delta$-1 subunits (11.1 h) than the $\alpha_2\delta$-2 subunits (2.4 h), in contrast to pregabalin (1.4 vs 1.4 h).

Fig. 3. Analgesic effects of mirogabalin and pregabalin in rats with partial sciatic nerve ligation on day 12 after surgery. (A) Time-course changes of paw withdrawal threshold. (B) Paw withdrawal threshold AUC. Mirogabalin besylate and pregabalin were orally administered (2 ml/kg). Control group received distilled water. Dose levels of test compounds are expressed as free form. Each value represents the mean ± S.E.M. (N = 12). $^{**}P < 0.01$: Significantly different from the control group by Dunnett's multiple comparison. Spearman's correlation coefficient reveals significant dose-response relationship in paw withdrawal threshold AUC_{0-8h} for mirogabalin (0.7860, $P < 0.0001$) and pregabalin (0.7081, $P < 0.0001$). According to the historical data, the paw withdrawal threshold in normal rats is approximately 12 g.

Fig. 4. Analgesic effects of mirogabalin and pregabalin in streptozotocin-induced diabetic rats. (A) Time-course changes of paw withdrawal threshold. (B) Paw withdrawal threshold AUC. Mirogabalin besylate and pregabalin were orally administered (2 ml/kg). Control group received distilled water. Dose levels of test compounds are expressed as free form. Each value represents the mean ±S.E.M. (N = 9 or 10). One rat in the group of pregabalin 10 mg/kg was excluded from the experiment due to administration error on Day 2. $^{*}P < 0.05$, $^{**}P < 0.01$: Significantly different from the control group by Dunnett's multiple comparison. Spearman's correlation coefficient reveals significant dose-response relationships in paw

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withdrawal threshold $AUC_{0–12h}$ as follows: mirogabalin (0.8669, $P < 0.0001$) and pregabalin (0.7545, $P < 0.0001$) on Day 1, mirogabalin (0.8867, $P < 0.0001$) and pregabalin (0.7255, $P < 0.0001$) on Day 3, mirogabalin (0.8724, $P < 0.0001$) and pregabalin (0.8123, $P < 0.0001$) on Day 5. ED_{50} values and their 95% confidence limits were estimated as follows: mirogabalin 4.4 (3.6–6.9) and pregabalin 26.8 (21.3–32.5) on Day 1, mirogabaline 3.1 (1.7–4.0) and pregabalin 22.4 (1.1–31.5) on Day 3, mirogabalin < 2.5 (95% confidence limits were not determined) and pregabalin 29.3 (25.3–34.5) on Day 5.

Fig. 5. Plasma concentrations of mirogabalin and pregabalin in streptozotocin-diabetic rats on Day 5. Mirogabalin besylate and pregabalin were orally administered (2 ml/kg). Dose levels and concentrations of test compounds are expressed as free form. Each value represents the mean ± S.E.M. (N = 3). Inset shows the dose-proportionality of C_{max} (μg/ml) and $AUC_{0–12h}$ (μg•h/ml).

Fig. 6. Effects of mirogabalin and pregabalin on rota-rod performance in rats. Mirogabalin besylate and pregabalin were orally administered (10 ml/kg). Control group received 0.5% methylcellulose solution. Dose levels of test compounds are expressed as free form. Each value represents the success rate of rota-rod performance (N = 8). *$P < 0.05$: Significantly different from the control group by Fisher's exact probability test. ED_{50} values for mirogabalin were 9.5 mg/kg (95% confidence limits were not determined) at 4 h and 9.4 mg/kg (5.4–17.4: 95% confidence limits) at 6 h after administration, respectively. ED_{50} values for pregabalin were 11.7 mg/kg (95% confidence limits were not determined) at 4 and 6 h after administration.

Fig. 7. Effects of mirogabalin and pregabalin on spontaneous locomotor activity in rats. Mirogabalin besylate and pregabalin were orally administered (10 ml/kg). Control group received 0.5% methylcellulose solution. Dose levels of test compounds are expressed as free form. Mirogabalin besylate

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was administered 6 h before the locomotor test. Pregabalin was administered 4 h before the locomotor test. Each value represents the mean ± S.E.M. (N = 8). *$P < 0.05$: Significantly different from the control group by Dunnett's multiple comparison. ED_{50} value for mirogabalin was 43.9 mg/kg (35.1–55.9: 95% confidence limits). ED_{50} value for pregabalin was 111.8 mg/kg (70.0–178.6: 95% confidence limits).

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TABLE 1

Binding parameters of ^{3}H-mirogabalin and ^{3}H-pregabalin for the $\alpha_2\delta$ subunits

Subtype	Parameter	Mirogabalin	Pregabalin
human $\alpha_2\delta$-1	K_d [nM]	13.5 (11.9–15.4)	62.5 (55.6–71.4)
	B_{max} [pmol/mg]	50.2 (46.1–55.3)	46.5 (42.5–51.5)
human $\alpha_2\delta$-2	K_d [nM]	22.7 (20.8–24.4)	125.0 (76.9–333.3)
	B_{max} [pmol/mg]	22.3 (21.0–23.7)	22.0 (15.2–45.9)
rat $\alpha_2\delta$-1	K_d [nM]	27.0 (24.4–29.4)	142.9 (125.0–200.0)
	B_{max} [pmol/mg]	47.8 (44.5–51.6)	38.2 (31.4–49.7)
rat $\alpha_2\delta$-2	K_d [nM]	47.6 (37.0–62.5)	166.7 (142.9–250.0)
	B_{max} [pmol/mg]	63.3 (52.2–82.3)	46.8 (37.8–62.7)

K_d: dissociation constant

B_{max}: maximum binding capacity

$K_d = -1/\text{slope}$, B_{max} = intercept of X-axis in Scatchard plot analysis

95% confidence limits in parenthesis

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TABLE 2

Dissociation kinetic parameters of ^{3}H-mirogabalin and ^{3}H-pregabalin for the $\alpha_2\delta$ subunits

Subtype	Parameter	Mirogabalin	Pregabalin
human $\alpha_2\delta$-1	K_{off} [h^{-1}]	0.0627 (0.0423–0.0831)	0.5051 (0.4817–0.5286)
	$t_{1/2}$ [h]	11.1 (8.3–16.4)	1.4 (1.3–1.4)
human $\alpha_2\delta$-2	K_{off} [h^{-1}]	0.2837 (0.2441–0.3233)	0.5103 (0.2603–0.7603)
	$t_{1/2}$ [h]	2.4 (2.1–2.8)	1.4 (0.9–2.7)
rat $\alpha_2\delta$-1	K_{off} [h^{-1}]	0.0798 (0.0629–0.0966)	0.4929 (0.4297–0.5561)
	$t_{1/2}$ [h]	8.7 (7.2–11.0)	1.4 (1.2–1.6)
rat $\alpha_2\delta$-2	K_{off} [h^{-1}]	0.3027 (0.2359–0.3695)	0.5266 (0.3937–0.6595)
	$t_{1/2}$ [h]	2.3 (1.9–2.9)	1.3 (1.1–1.8)

K_{off}: dissociation rate constant

$t_{1/2}$: dissociation half-life

95% confidence limits in parenthesis

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TABLE 3

Pharmacokinetic parameters of mirogabalin and pregabalin in streptozotocin-induced diabetic rats

Drug	Dose	Day 1		Day 5	
		C_{max}	$AUC_{0\text{-}12h}$	C_{max}	$AUC_{0\text{-}12h}$
	(mg/kg)	(μg/ml)	(μg•h/ml)	(μg/ml)	(μg•h/ml)
Mirogabalin	2.5	0.5 ± 0.1	2.6 ± 0.5	0.5 ± 0.1	1.9 ± 0.0
	5	1.5 ± 0.0	7.2 ± 0.8	1.0 ± 0.1	5.2 ± 0.7
	10	3.2 ± 0.0	10.9 ± 0.5	2.5 ± 0.2	15.1 ± 2.7
Pregabalin	10	7.0 ± 1.9	42.4 ± 0.4	6.8 ± 0.5	46.3 ± 6.4
	20	11.6 ± 3.3	69.4 ± 7.0	14.9 ± 0.7	94.2 ± 17.6
	40	20.3 ± 0.5	148.7 ± 13.7	26.7 ± 2.7	165.3 ± 16.7

Mirogabalin besylate and pregabalin were orally administered (2 ml/kg).

Dose levels and concentrations of test compounds are expressed as free form.

Each value represents the mean ± S.E.M. (N = 3).

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TABLE 4

CNS side effects profile of mirogabalin and pregabalin in rats

		Mirogabalin	Pregabalin
Analgesic ED_{50}	STZ diabetic model	4.4 mg/kg	26.8 mg/kg
CNS side effect ED_{50}	Rota-rod performance	9.4 mg/kg	11.7 mg/kg
	Locomotor activity	43.9 mg/kg	111.8 mg/kg
Safety index	Rota-rod performance	2.1	0.4
	Locomotor activity	10.0	4.2

Dose levels of test compounds are expressed as free form.

Safety index was obtained by calculating the ratio between side effect ED_{50} (rota-rod ED_{50} or locomotor ED_{50}) and analgesic ED_{50} (on Day 1 in STZ diabetic model).

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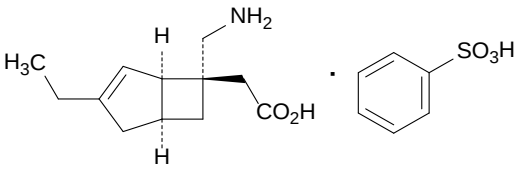

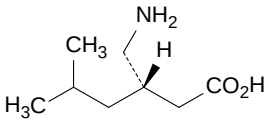

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Fig. 1

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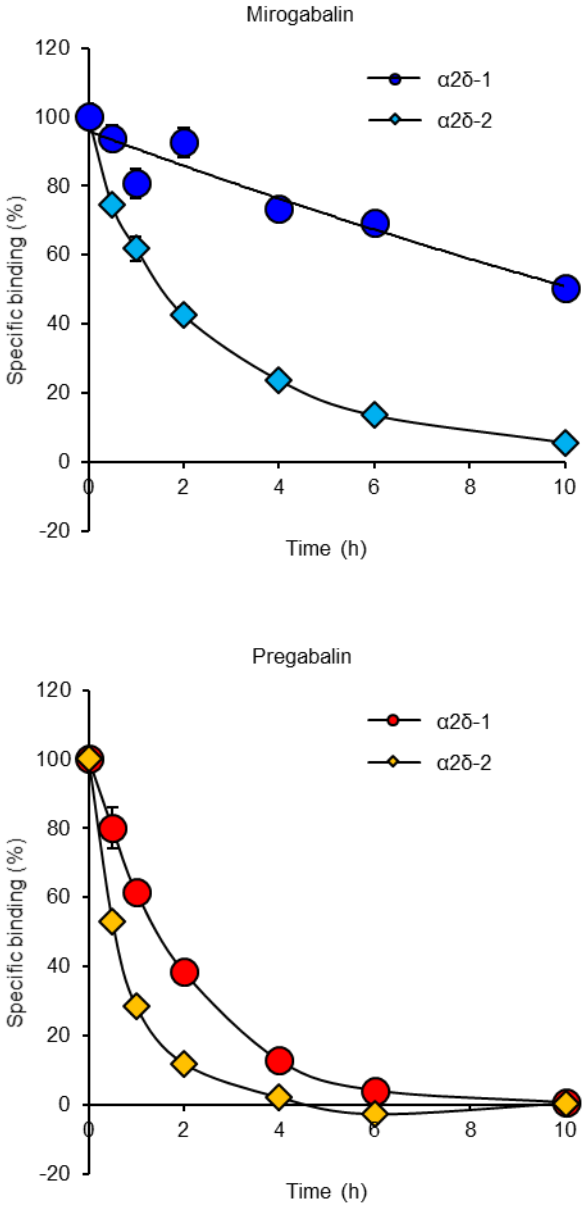

Fig. 2

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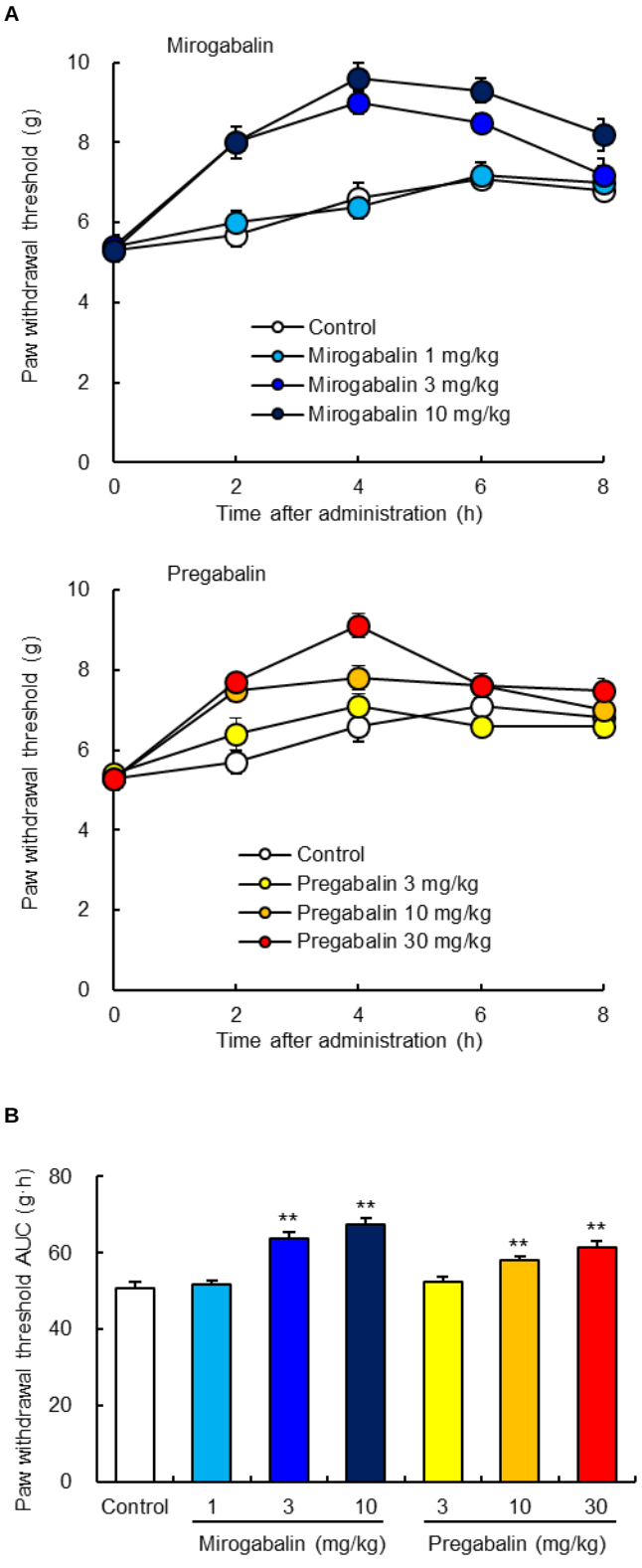

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Fig. 3

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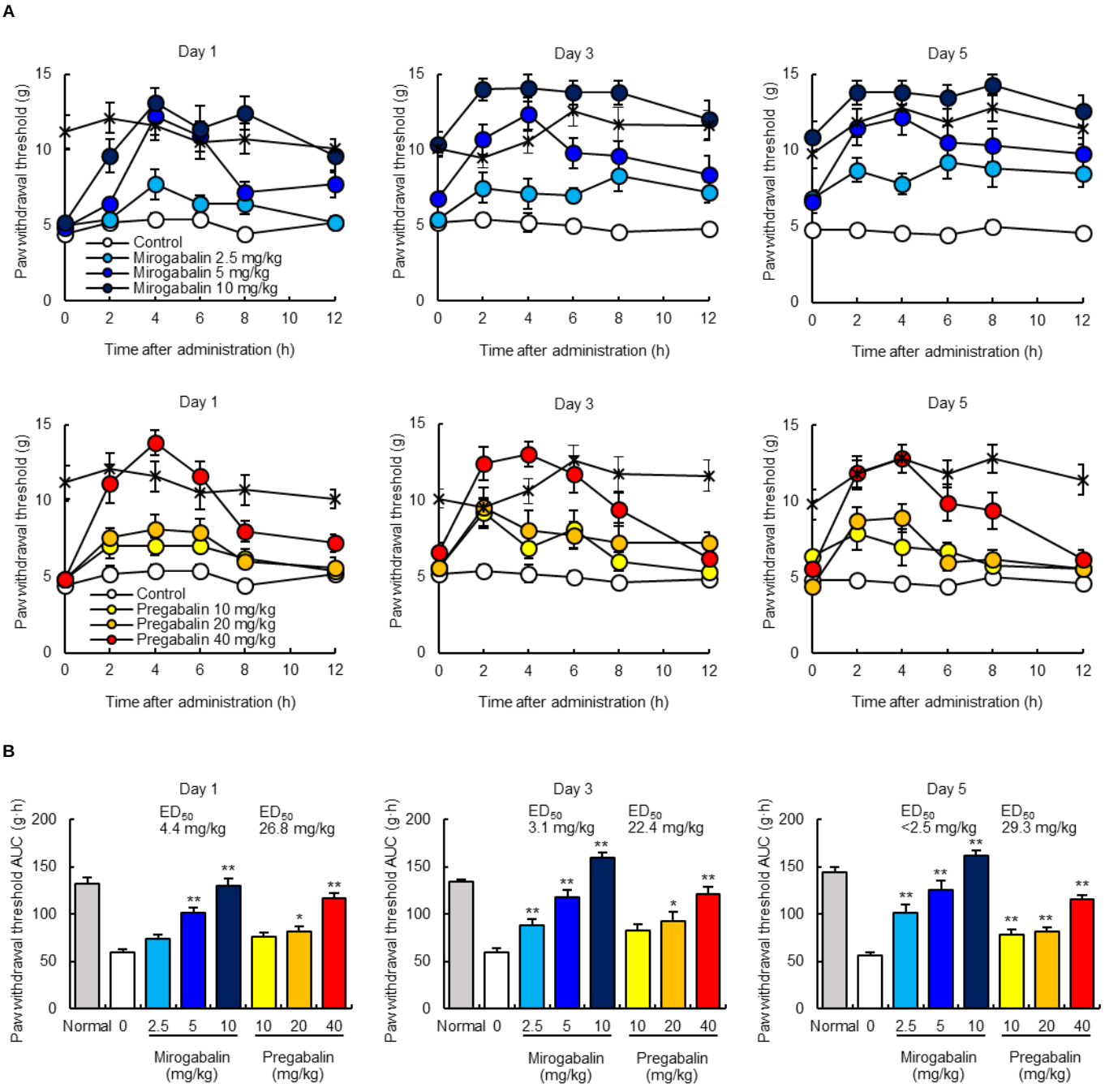

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Fig. 4

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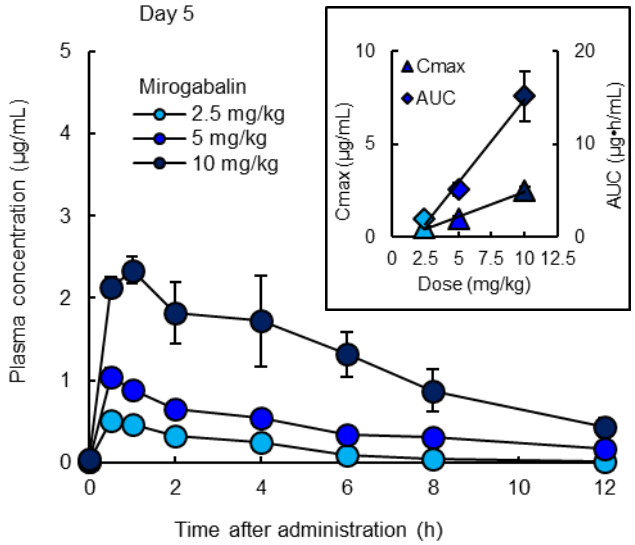

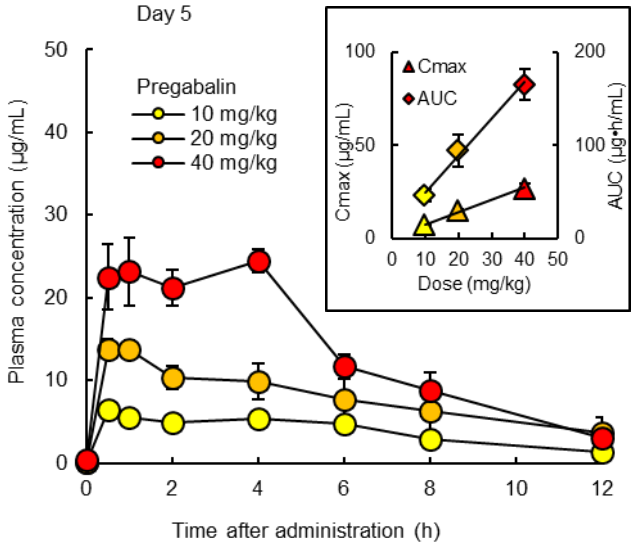

Fig. 5

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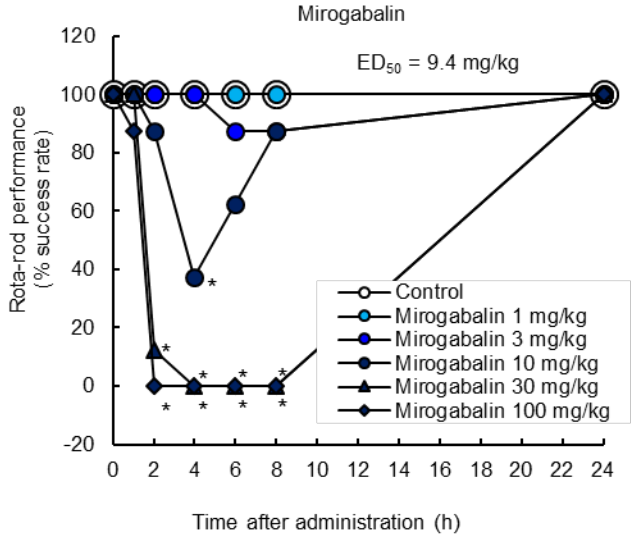

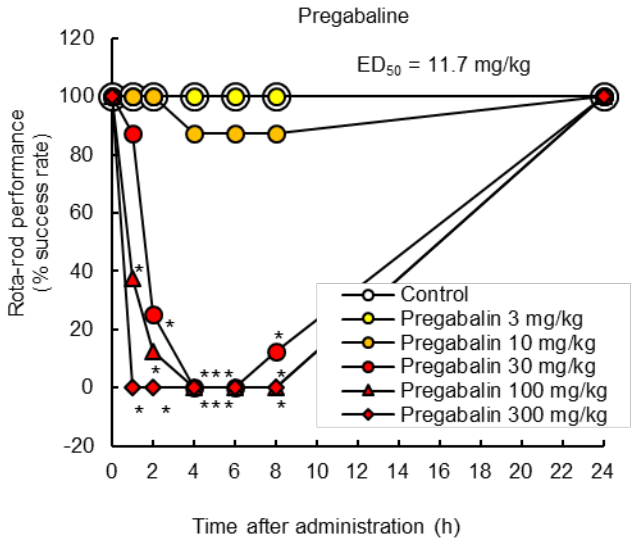

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Fig. 6

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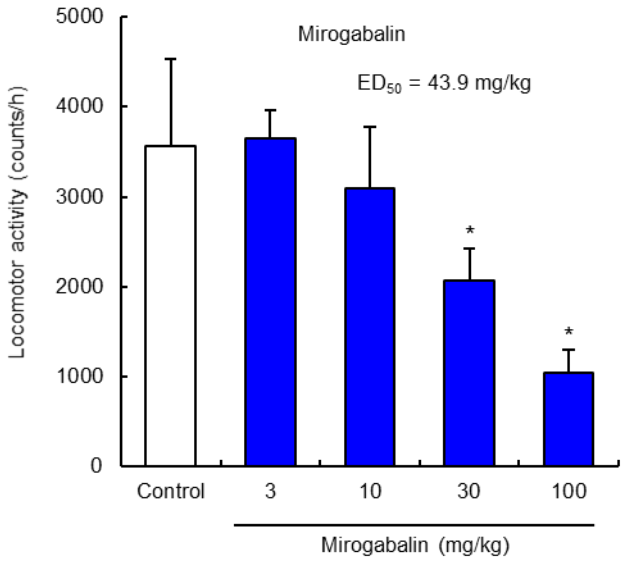

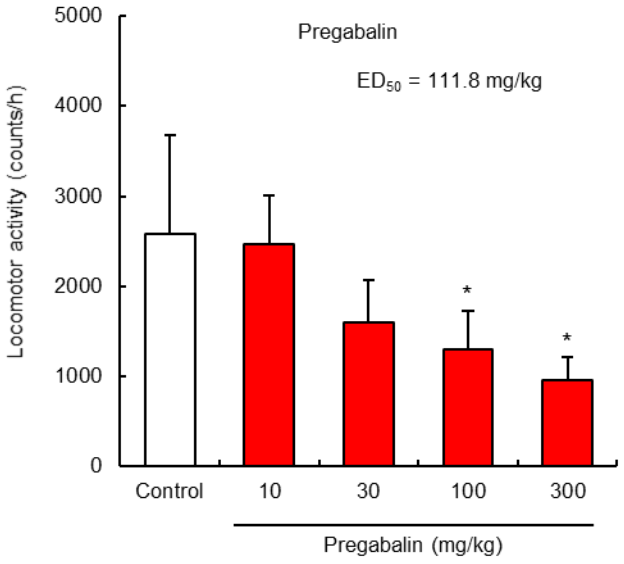

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Fig. 7