Binding Characteristics and Analgesic Effects of Mirogabalin, a Novel Ligand for the α2δ Subunit of Voltage-Gated Calcium Channels

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ABSTRACT

Mirogabalin ([1R,5S,6S)-6-(aminomethyl)-3-ethylbicyclo[3.2.0]hept-3-en-6-yl]acetic acid), a novel ligand for the α2δ 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 with those of pregabalin, a standard α2δ ligand. Mirogabalin showed potent and selective binding affinities for the α2δ subunits, while having no effects on 186 off-target proteins. Similar to pregabalin, mirogabalin did not show clear subtype selectivity (α2δ-1 vs. α2δ-2) or species differences (human vs. rat). However, in contrast to pregabalin, mirogabalin showed greater binding affinities for human α2δ-1, human α2δ-2, rat α2δ-1, and rat α2δ-2 subunits; further, it had a slower dissociation rate for the α2δ-1 subunit than the α2δ-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 α2δ subunits, and slower dissociation rates for the α2δ-1 subunit than the α2δ-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.

Introduction

Gabapentinoids, such as pregabalin and gabapentin, are selective ligands for the α2δ 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., 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 guidelines recommend pregabalin and gabapentin as the first-line drugs for the treatment of neuropathic pain (Argoff et al., 2006; Dworkin et al., 2007, 2013; Finnerup and Jensen, 2007; Attal et al., 2010; Bril et al., 2011; 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 \( \alpha_\delta-1 \) subunit of voltage-gated calcium channels contributes to analgesic effects (Field et al., 2006; Li et al., 2006), whereas the \( \alpha_\delta-2 \) subunit contributes to CNS side effects (Barclay et al., 2001; Brill et al., 2004), suggesting that ligand selectivity for \( \alpha_\delta-1 \) and \( \alpha_\delta-2 \) might result in different clinical outcomes.

Mirogabalin ([1R,5S,6S]-6-(aminomethyl)-3-ethylbicyclo[3.2.0]-hept-3-en-6-yl)acetic acid) is a novel ligand for the \( \alpha_\delta \) 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 with those of pregabalin.

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 dimethylsulfoxide. The dose levels of test compounds are expressed as free form. All other reagents were of analytical grade and were obtained from conventional commercial sources. The chemical structures of mirogabalin besylate and pregabalin are shown in Fig. 1.

Cell Membranes

The cell membrane fraction containing each \( \alpha_\delta \) subunit was prepared from the 293A stable cell line expressing each \( \alpha_\delta \) subunit, as described in a previous report (Jee et al., 1996). Briefly, 293A cells (Thermo Fisher Scientific, Waltham, MA) were cotransfected with \( pPURO \) (Clontech Laboratories, Inc., Mountain View, CA) by lipofection. The transfected 293A cells were diluted and spread to 150-mm dishes. After 10 days in culture, the cell colonies were picked up and selected by a scraper, and the membrane fraction was collected by centrifugation. The pellet of membrane was washed with binding assay buffer (0.01 M HEPES, pH 7.5, and 0.1 M NaCl) three times by repetition of suspension and centrifugation. Total protein concentration of membrane fractions were determined by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). The total protein concentrations of human \( \alpha_\delta-1 \) subunit–expressing 293A cell membrane, human \( \alpha_\delta-2 \) subunit–expressing 293A cell membrane, rat \( \alpha_\delta-1 \) subunit–expressing 293A cell membrane, and rat \( \alpha_\delta-2 \) subunit–expressing 293A cell membrane were 11.2, 10.7, 9.8, 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-hour light/dark cycle (lights on from 7:00 AM to 7:00 PM), 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, Labor and Welfare (Notification Number 0601001 of the Science Bureau, Japanese Ministry of Health, Labor 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 \( \alpha_\delta \) Subunits

Saturation Assay. The cell membranes prepared as described above were diluted with binding assay buffer (0.01 M HEPES, pH 7.5, and 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 96-U plate (MS-3296U; Sumitomo Bakelite Co., Ltd., Tokyo, Japan) and incubated for 4 hours 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 three times with 400 \( \mu l \) of binding assay buffer and dried overnight at room temperature. The radioactivity of the dried filter picked up from each well was counted by a liquid scintillation counter (PerkinElmer Inc., Waltham, MA) after being immersed in 4 ml of Pico-Fluor 40 (PerkinElmer) in a glass vial. Nonspecific binding was defined as the residual binding in the presence of 100 \( \mu M \) mirogabalin or 200 \( \mu M \) 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 hours at room temperature, as described above. After that, the dissociation reaction was initiated by adding 2 \( \mu l \) of the corresponding unlabeled compound solution (10 nM mirogabalin or 20 nM pregabalin), and the reaction mixture was incubated for 0 hours (immediately), and for 0.5, 1, 2, 4, 6, and 10 hours at room temperature (\( N = 4 \)). After incubation, the membrane-bound radioligand was recovered by filtration, and the radioactivity was assayed. For the Western blotting assay, anti-\( \alpha_\delta-1 \) antibody (C5105; Sigma-Aldrich, St. Louis, MO) and anti-\( \alpha_\delta-2 \) antibody (sc-34768; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used for the detection of \( \alpha_\delta-1 \) and \( \alpha_\delta-2 \), respectively. The cells harvested by scrapers were lysed by sonication on ice, and the membrane fraction was collected by centrifugation. The pellet of membrane was washed with binding assay buffer (0.01 M HEPES, pH 7.5, and 0.1 M NaCl) three times by repetition of suspension and centrifugation. Total protein concentration of membrane fractions were determined by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). The total protein concentrations of human \( \alpha_\delta-1 \) subunit–expressing 293A cell membrane, human \( \alpha_\delta-2 \) subunit–expressing 293A cell membrane, rat \( \alpha_\delta-1 \) subunit–expressing 293A cell membrane, and rat \( \alpha_\delta-2 \) subunit–expressing 293A cell membrane were 11.2, 10.7, 9.8, and 5.7 mg/ml, respectively.

Fig. 1. Chemical structures of mirogabalin besylate (top) and pregabalin (bottom).
were determined in the follow-up assays. The area under the curve (AUC) of the 8-hour paw withdrawal threshold was measured at 0 hours (before administration), and at 2, 4, 6, and 8 hours after treatment groups. The rats received the test compound or vehicle (control) orally, and the paw withdrawal threshold was measured at approximately one-half to one-third of the sciatic nerve was tightly ligated femoral skin was incised and the sciatic nerve was exposed. Approximately one-half to one-third of the sciatic nerve was tightly ligated with surgical 4-0 silk thread. Twelve days after PSL, the paw sutured with surgical 8-0 silk thread. The surgical area was sterilized with kanamycin (Meiji Seika Pharma Co., Ltd., Tokyo, Japan) and was used for the validation of the Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS) method.

In Vitro Off-Target Pharmacological Profile

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, seven 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 IC50 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 (Escan, Mylan Inc., Osaka, Japan), the left femoral skin was incised and the sciatic nerve was exposed. Approximately one-half to one-third 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 (model 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 seconds, at 0.5 g/step). PSL rats with a 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 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 hours (before administration), and at 2, 4, 6, 8, and 12 hours after the first administration on day 1, day 3 and day 5. The von Frey test was performed at 0 hours (before administration), and at 2, 4, 6, 8, and 12 hours after the first administration on day 1, day 3 and day 5. The AUC of the 12-hour paw withdrawal threshold was calculated by the trapezoidal method. Satellite groups (three animals per group) were set aside for a pharmacokinetics (PK) evaluation. Blood samples were collected from the tail vein using heparinized microhematocrit capillary tubes, and the plasma concentrations of mirogabalin and pregabalin were determined by the validated liquid chromatography-tandem mass spectrometry method.

Rota-Rod Performance in Rats

Ninety-six male Fischer rats were divided into groups of eight. Rats able to walk on the rota-rod (6 rpm, 9 cm in diameter; model KN-75; Natsume Seisakusho Co., Ltd., Tokyo, Japan) for 3 minutes 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 seconds in three trials. The rota-rod test was performed at 0 hours (before administration), and at 2, 4, 6, 8, and 12 hours after the first administration on day 1, day 3 and day 5. The AUC of the 12-hour paw withdrawal threshold was calculated by the trapezoidal method. Satellite groups (three animals per group) were set aside for a pharmacokinetics (PK) evaluation. Blood samples were collected from the tail vein using heparinized microhematocrit capillary tubes, and the plasma concentrations of mirogabalin and pregabalin were determined by the validated liquid chromatography-tandem mass spectrometry method.

| Subtype | Parameter | Mirogabalin | Pregabalin |
|---------|-----------|-------------|------------|
| Human α6β-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 α2β-2 | \( B_{max} \) [pmol/mg] | 22.7 (20.8–24.4) | 125.0 (76.9–333.3) |
| | \( B_{max} \) [pmol/mg] | 22.3 (21.9–23.7) | 22.0 (15.2–45.9) |
| Rat α2β-1 | \( K_d \) [nM] | 27.0 (24.4–28.4) | 142.9 (125.0–200.0) |
| | \( B_{max} \) [pmol/mg] | 47.6 (44.5–51.6) | 38.2 (31.4–49.7) |
| Rat α2β-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) |

\( t_{1/2} \), dissociation half-life.

TABLE 2

Dissociation kinetic parameters of \(^3^H\)-mirogabalin and \(^3^H\)-pregabalin for the α2β subunits

The 95% confidence limits are in parentheses.

| Subtype | Parameter | Mirogabalin | Pregabalin |
|---------|-----------|-------------|------------|
| Human α6β-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 α2β-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 α2β-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 α2β-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) |

\( K_d \) = -1/slope, \( B_{max} \) = intercept of x-axis in Scatchard plot analysis. The 95% confidence limits are shown in parentheses.

Streptozotocin-Induced Diabetic Model in Rats

Seventy diabetic male Brown Norway rats were divided into groups of 10. Ten normal male rats were used as the nondiabetic 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 success rate, 59%). Sixteen weeks after the STZ injection, the paw withdrawal threshold in response to mechanical stimulation was determined using von Frey filaments (North Coast Medical Inc., Gilroy, CA). STZ rats with a 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 hours (before administration), and at 2, 4, 6, 8, and 12 hours after the first administration on day 1, day 3 and day 5. The AUC of the 12-hour paw withdrawal threshold was calculated by the trapezoidal method. Satellite groups (three animals per group) were set aside for a pharmacokinetics (PK) evaluation. Blood samples were collected from the tail vein using heparinized microhematocrit capillary tubes, and the plasma concentrations of mirogabalin and pregabalin were determined by the validated liquid chromatography-tandem mass spectrometry method.
conducted at 0 hours (before administration), and at 2, 4, 6, 8, and 24 hours after administration.

**Spontaneous Locomotor Activity in Rats**

Eighty male Fischer rats were divided into groups of eight. After oral administration of the test compound or vehicle (control), locomotor activity was measured for 1 hour using the SUPERMEX system (model 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 hours for mirogabalin besylate and at 4 hours for pregabalin.

**Statistical Analysis**

For the in vitro saturation assay and dissociation kinetic assay, the $B_{max}$, dissociation constant ($K_d$), dissociation rate constant ($K_{off}$), and dissociation half-life, including their 95% confidence limits, were calculated. The statistical analysis was performed using Dunnett’s multiple-comparison test. Spearman’s correlation coefficient revealed a significant dose-response relationship in AUC$_{0-8}$ values 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.
calculated. For paw withdrawal threshold AUC and locomotor activity, the statistical analysis was performed using Dunnett’s multiple-comparison test. 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 \( P < 0.05 \). The ED\textsubscript{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 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. The 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...
The dissociation half-life of pregabalin from the human $\alpha_2\delta$-1 and $\alpha_2\delta$-2 subunits were estimated to be 11.1 and 2.4 hours, respectively. Further, neither compound exhibited subtype selectivity (human vs. rat). 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 with 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. Figure 2 shows dissociation curves for the human $\alpha_2\delta$-1 and human $\alpha_2\delta$-2 subunits. The dissociation half-lives of mirogabalin from the human $\alpha_2\delta$-1 and $\alpha_2\delta$-2 subunits were estimated to be 11.1 and 2.4 hours, 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 hours. The dissociation half-lives of mirogabalin from the rat $\alpha_2\delta$-1 and $\alpha_2\delta$-2 subunits were estimated to be 8.7 and 2.3 hours, respectively. The dissociation half-lives of pregabalin from the rat $\alpha_2\delta$-1 and $\alpha_2\delta$-2 subunits were estimated to be 1.4 and 1.3 hours, 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**

Figure 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 AUC_{0-12 hours} values in a dose-dependent manner. The effect of mirogabalin peaked at 4 hours after administration and remained there until 6 or 8 hours after administration. The effect of pregabalin peaked at 4 hours after administration and returned to the vehicle control level after 6 hours.

**STZ-Induced Diabetic Model in Rats**

Figure 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 AUC_{0-12 hours} values in a dose-dependent manner. The effects of mirogabalin and pregabalin peaked at 4 hours 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, whereas 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.

Figure 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_{0-12 hours} values. Table 3 summarizes the PK parameters on day 1 and day 5. The $C_{max}$ and AUC_{0-12 hours} values of mirogabalin and pregabalin increased nearly dose proportionally. There were no apparent changes in these parameters for mirogabalin and pregabalin, even after repeated dosing.

**Rota-Rod Performance in Rats**

Figure 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
hours after administration, and the ED50 values were estimated to be 9.5 and 9.4 mg/kg, respectively, at 4 and 6 hours after administration. 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 hours after administration, and the ED50 value was considered to be 11.7 mg/kg. All animals recovered 24 hours after the administration of mirogabalin and pregabalin, at all dosage levels.

### Spontaneous Locomotor Activity in Rats

Figure 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 ED50 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 ED50 values (rota-rod ED50 or locomotor ED50) and analgesic ED50 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 mirogabalin were superior to those of pregabalin in both tests.

### Discussion

The α2δ subunits are multifunctional and have been reported to affect calcium channel trafficking as well as the biophysical properties of calcium channel currents (Dolphin, 2012, 2013). Increased expression of α2δ-1 mRNA and α2δ-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, 2010; Boroujerdi et al., 2011). In addition, knockdown of α2δ-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 α2δ-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 α2δ-1 knockout mice have been reported to show markedly reduced behavioral sensitivity to mechanical and cold stimuli. The knockout mice also showed a delayed development of mechanical hypersensitivity after FSL and loss of the analgesic effect of pregabalin (Patel et al., 2013). Knockin mice expressing a mutant α2δ-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 the α2δ-1 subunit has an important role in neuropathic pain states and that the analgesic effects of gabapentinoids are mediated through the α2δ-1 subunit. In contrast, the α2δ-2 subunit is dominantly expressed in the cerebellar Purkinje cells, and mutant mice with a α2δ-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 α2δ-2 mutations have been reported (Edvardson et al., 2013; Pippucci et al., 2013). These distinct roles of α2δ-1 and α2δ-2 subunits suggest that ligand selectivity for α2δ-1 and α2δ-2 might bring about separate analgesic effects and CNS side effects.

In the present study, mirogabalin specifically bound to α2δ subunits with high affinity at two-digit nanomolar concentrations and had no effects on a total of 186 off-target proteins at three orders of higher concentration (50 μM). The binding affinities of mirogabalin for the human α2δ-1, human α2δ-2, rat α2δ-1, and rat α2δ-2 subunits were greater than those of pregabalin, and neither compound showed subtype selectivity (α2δ-1 vs. α2δ-2) or species differences (human vs. rat). Interestingly, although the subtype selectivity of mirogabalin is not significant in Kd values, mirogabalin showed longer dissociation half-lives against the α2δ-1 subunit than the α2δ-2 subunit both in human and rat. On the other hand, pregabalin showed equal dissociation half-lives from the α2δ-1 subunit and the α2δ-2 subunit in both human and rat. The binding affinity of mirogabalin and pregabalin for the α2δ-3 or α2δ-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 the α2δ-3 or α2δ-4 subunit have not yet been reported.

### Table 3

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

| Drug     | Dose (mg/kg) | Cmax (μg/ml) | AUC0-12 hours (μg h/ml) | Dose (mg/kg) | Cmax (μg/ml) | AUC0-12 hours (μg h/ml) |
|----------|--------------|--------------|-------------------------|--------------|--------------|-------------------------|
| Mirogabalin | 2.5          | 0.5 ± 0.1    | 2.6 ± 0.5               | 5            | 1.5 ± 0.0    | 7.2 ± 0.8               |
|          | 10           | 3.2 ± 0.0    | 10.9 ± 0.5              | 10           | 7.0 ± 1.9    | 42.4 ± 0.4              |
|          | 20           | 11.6 ± 3.3   | 68.9 ± 7.0              | 20           | 11.6 ± 3.3   | 68.9 ± 7.0              |
|          | 40           | 20.3 ± 0.5   | 148.7 ± 13.7            | 40           | 20.3 ± 0.5   | 148.7 ± 13.7            |

| Drug     | Dose (mg/kg) | Cmax (μg/ml) | AUC0-12 hours (μg h/ml) |
|----------|--------------|--------------|-------------------------|
| Pregabalin | 10           | 0.5 ± 0.1    | 2.6 ± 0.5               |
|          | 20           | 1.5 ± 0.0    | 7.2 ± 0.8               |
|          | 30           | 3.2 ± 0.0    | 10.9 ± 0.5              |
|          | 100          | 7.0 ± 1.9    | 42.4 ± 0.4              |
|          | 300          | 11.6 ± 3.3   | 68.9 ± 7.0              |
|          | 600          | 20.3 ± 0.5   | 148.7 ± 13.7            |

ED50 values were estimated to be 9.5 and 9.4 mg/kg, respectively, at 4 and 6 hours after administration. The maximum effect was observed 4–6 hours after administration, and the ED50 value was considered to be 11.7 mg/kg. All animals recovered 24 hours after the administration of mirogabalin and pregabalin.
In typical experimental neuropathic pain models, such as PSL rats and STZ-induced diabetic rats, mirgabalin showed more potent and longer lasting analgesic effects than pregabalin. The greater binding affinity of mirgabalin for the $\alpha_{2d}$ subunit is considered to contribute to its potent analgesic effects. Interestingly, unlike pregabalin, the analgesic effects of mirgabalin were enhanced by repeated administration in STZ-induced diabetic rats. In the mirgabalin treatment groups, the paw withdrawal threshold AUCs increased day by day without increases in the AUCs of plasma drug concentrations. Specifically, at 10 mg/kg mirgabalin, 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 mirgabalin for the $\alpha_{2d}$ 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 with which to determine the duration of pharmacological effects and target selectivity in vivo (Copeland et al., 2006). In safety pharmacological evaluations, mirgabalin and pregabalin dose-dependently inhibited rota-rod performance and
lack of safety margin. These effects are the class effects of gabapentinoids and have been suggested to be related to the 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 α2δ-1 subunit plays an important role in the analgesic effects of gabapentinoids, whereas the α2δ-2 subunit is related to CNS side effects. Mirogabalin had a longer dissociation half-life from the α2δ-1 subunit than the α2δ-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 a 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/d, given either once or twice daily with or without titration.

In conclusion, mirogabalin shows potent and selective binding affinities for the human and rat α2δ subunits, and a slower dissociation rate for the α2δ-1 than for the α2δ-2 subunit. It shows potent and long-lasting analgesic effects in rat models for neuropathic pain and a 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

Participated in research design: Arakawa, Matsuda, Yamamura, Kai, and Kitanо. Conducted experiments: Domon, Arakawa, Inoue, Matsuda, Takahashi, Yamamura, Kai, and Kitanо. Performed data analysis: Domon, Yamamura, and Kitanо. Wrote or contributed to the writing of the manuscript: Matsuda, Yamamura, Kai, and Kitanо.

TABLE 4
CNS side effects profile of mirogabalin and pregabalin in rats

|                  | Mirogabalin | Pregabalin |
|------------------|-------------|------------|
|                  | STZ diabetic model | Rota-rod ED50 | Motor activity | Rota-rod ED50 | Locomotor activity |
| Analgesic ED50   | 4.4 mg/kg    | 26.8 mg/kg  |
| CNS side-effect ED50 | 9.4 mg/kg    | 11.7 mg/kg  |
| Safety index     | 2.1         | 0.4        |

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