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KST5468, a new T-type calcium channel antagonist, has an antinociceptive effect on inflammatory and neuropathic pain models

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ABSTRACT

The T-type Ca^{2+} channel is a low-voltage-activated Ca^{2+} channel related to nociceptive stimuli. Increases in Ca^{2+} due to calcium channel activation enhance pain sensitivity through both peripheral and central pain pathways. We have developed a novel compound, KST5468, which is a T-type calcium channel antagonist. The new synthetic compound may have an antinociceptive effect, and thus we evaluated KST5468 as a putative analgesic in a hot plate test, a formalin test, and two neuropathic pain models. KST5468 caused a significant increase in latency in the hot plate test at 30 min after a 10 mg/kg peritoneal injection of the compound. Interestingly, in the second phase of formalin test, KST5468 decreased pain behaviors in a dose-dependent manner. Moreover, in two neuropathic pain models induced by chronic constriction and spared nerve injury, KST5468 significantly increased the mechanical pain threshold. Using immunohistochemistry, expression of two well known pain-related molecular markers, c-Fos and calcitonin gene-related peptide (CGRP), and phosphorylated extracellular signal-related kinase (p-ERK) were found to be decreased in the laminae I–II layers of the ipsilateral L4–L5 spinal dorsal horn in KST5468 treated mice. Taken together, the results of this study suggest that KST5468 may be an effective antinociceptive agent for neuropathic pain.

1. Introduction

The pathological pain such as inflammatory or neuropathic pain is a challenging subject that requires further studies and research. Peripheral nerve injury and inflammation cause abnormal ectopic discharges from the injury sites as well as increased central input from injured primary afferent fibers (Kim et al., 2001). Spontaneous pain, allodynia, and hyperalgesia are commonly observed in inflammatory and neuropathic pain conditions in both animal models and patients with chronic pain (Blackburn-Munro, 2004). Moreover the damaged primary afferent fibers appear to have altered excitability and conduction patterns during the initiation and maintenance phases of pathological pain. In addition, these altered patterns reflect changes in multiple ion channels, such as their density or operating characteristics. Differential alterations of specific ion channels due to the damaged afferent fibers may be important determinants of primary afferent discharge and conduction (Gold et al., 2003).

Local Ca^{2+} concentrations play a critical role in intracellular signal transduction and also they control various cellular functions. Recent evidence suggests that T-type Ca^{2+} channels, particularly in the neurons of the dorsal root ganglia and dorsal horn, can enhance nociceptive inputs as well as regulate changes in pain thresholds in a number of different animal models (Ikeda et al., 2003). Moreover, many researchers who have studied diverse T-type Ca^{2+} currents in isolated sensory neurons suggested that these channels might be selectively expressed by nociceptive neurons (Lawson, 2002; Shin et al., 2003). The T-type Ca^{2+} channels are sub-typed as α1G, α1H, and α1I (Catterall et al., 2005); these subtypes are expressed concomitantly or differentially throughout the nervous system (Shin et al., 2008).

Calcium channel inhibitors have been used extensively for treating hypertension, epilepsy, and even neuropathic pain. Compared with Ca^{2+} channel blockers specific for other subtypes, T-type Ca^{2+} channel blockers may have decreased incidence of side effects such as atrioventricular blockage and negative inotropism (Opie, 1988). Mibefradil, a relatively selective T-type Ca^{2+} channel blocker (Coulter et al., 1989), was the first marketed T-type Ca^{2+} channel blocker,

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however, it was later rejected because of its pharmacokinetic interactions with other drugs (Billups and Carter, 1998; SoRelle, 1998). Among the three types of T-type Ca^{2+} channels, the α1G subtype may have a nociceptive role in persistent inflammatory pain and may be involved especially in the development of neuropathic pain at the spinal level (Shin et al., 2008). Prior to the experiment, we tested the most plausible compounds from a library of newly synthesized piperazinealkylisoxazole derivatives (Jung et al., 2004) *in vitro* and in vivo. Screening for selectivity over hERG (human ether-a-go-go-related gene) channel binding affinity was done in accordance with the current regulatory guidelines (ICH S7B) to lower the risk of potentially life-threatening cardiac arrhythmias (Lindgren et al., 2008; Towart et al., 2009). After the screening test in vitro, we also injected several selected compounds intraperitoneally and evaluated behavioral changes in a formalin test in mice. Finally, based on preliminary results, KST5468 was selected for a further evaluation using in vivo pain models in this study (Fig. 1).

In this study, we also examined the effect of KST5468 on noxious and inflammatory pain using behavioral tests. In addition, we compared the expression of c-Fos, calcitonin gene-related peptide (CGRP), and phosphorylated extracellular signal-related kinase (p-ERK), which is closely associated with central and peripheral pain sensitization after administrating KST5468 in mice.

2. Materials and methods

2.1. Animals

Five Male ICR mice (7–8 weeks, 25–30 g, Orient Bio Inc., Gyeonggido, Republic of Korea) were housed per cage covered with soft bedding. Mice were allowed free access to food and water in a temperature controlled room (20 °C) with a 12/12 hour day/night cycle. Mice were allowed to be accustomed to the environment for at least 3 days prior to their usage in behavior tests. The mice were euthanized with carbon dioxide method after the experiments (Conlee et al., 2005). All experiments were performed according to the Guidelines on Ethical Standards for Investigation of Experimental Pain in Animals (Zimmermann, 1983). Additionally, the study protocol was approved by the Institutional Animal Care and Use Committee of Seoul National University.

2.2. Drugs

KST5468, which was chemically synthesized by our laboratory (Pae AN et al.) for this study was dissolved in 5% DMSO (1, 5, and 10 mg/kg) and gabapentin (100 mg/kg) was dissolved in saline. Gabapentin was used as a positive control in neuropathic pain models. Tramadol was used as a positive control in the hot plate test. All drugs were administered intraperitoneally (150 μl) except during pharmacokinetic study in which the drugs were injected both intraperitoneally and intravenously (150 μl) via the tail vein. Injection dose (10 mg/kg) was selected on the basis of the formalin test.

2.3. Pharmacokinetic study

KST5468 (dissolved in N-methylpyrrolidone:Tween80:distilled water = 1:2:7; v/v/v) was administered at a dose of 10 mg/kg (total injection volume of approximately 0.15 ml) via the tail vein of each ICR mouse. At 0 (to serve as a control), 5, 15, 30, 60, 120, 240, 360 and 480 min (n = 3 at each blood sampling time), blood was collected as much as possible via cardiac puncture. Blood samples were centrifuged immediately and a 50 μl aliquot of plasma sample was stored in a −70 °C freezer until high performance liquid chromatography-tandem mass spectrometry analysis of KST5468 could be performed. After the blood sampling, exsanguinated mice were sacrificed and their brain tissue was harvested. The brain tissue was weighed and homogenized with 4 volume of distilled water using a tissue homogenizer (Ultra-Turrax, T25, Janke & Kunkel, IKA-Labortechnik, Staufen, Germany). A 100 μl aliquot of brain tissue homogenate was stored in a −70 °C freezer until high performance liquid chromatography-tandem mass spectrometry analysis of KST5468 cold be performed. KST5468 solution (the same solution used in the intravenous study) at a dose of 10 mg/kg was intraperitoneally administered to mice. After the injection, blood and brain tissue samples were collected and handled with the similar method as described in the above intravenous studies. The lower limits of quantitation of KST5468 in plasma and brain tissue homogenates were 10 ng/ml and 10 ng/g, respectively. The linear regression of the calibration curves was fitted by a 1/x weighting. The value of coefficients of determination (R^2) ranged from 0.997 to 0.999.

Pharmacokinetic parameters were determined by a non-compartmental analysis using WinNonlin® (Pharsight Corporation, Mountain View, CA) program. The total area under the plasma concentration–time curve from time zero to infinity (AUC) was calculated by the trapezoidal rule-extrapolation method. Standard methods (Perrier and Gibaldi, 1974, 1982) were used to calculate the following pharmacokinetic parameters; the time-averaged total body clearance (CL), total area under the first moment of plasma concentration–time curve from time zero to time infinity (AUMC), terminal half-life, mean residence time (MRT), and apparent volume of distribution at steady state (V_{ss}).

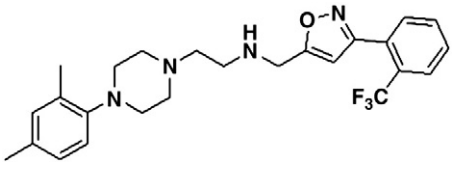

Fig. 1. The structure of KST5468.

2.4. Formalin test

The formalin test was performed to determine the effect of KST5468 on inflammatory pain response (Hunskaar and Hole, 1987; Rosland et al., 1990; Tjolsen et al., 1992). Briefly, mice were housed individually in plexiglass cylinders (20 cm in diameter and 35 cm in height) with a transparent floor and a mirror placed under the floor to assist with observation. Mice were habituated in these housing units for 20–30 min prior to drug injection using a 31 G needle. Mice were observed for 15 min in order to monitor whether or not the test compounds affected the animal's motor behavior. The locomotor activity was assessed by subjective judgment compared to naïve mice in open field chamber (40 × 20 × 20 cm). When behavioral changes were confirmed to be absent, mice were treated with an intraplantar injection of formalin (5% in distilled water 20 μl) in the left hindpaw using a 31 G needle. Following the injection of formalin, the duration of paw-licking was measured for 0–5 min (first phase) and 20–40 min (second phase). For each group analyzed, the number of animals was greater than 7.

2.5. Rotarod test

Prior to rotarod test, the mice were trained for two days. The rotarod (Panlab, Spain), consisting of a non-slippery plastic rod (30 mm diameter) and 4 lines (50 mm wide), was selected to a run mode (16 rpm) for over 1 min. The trained mice were subject to run on the rod for at least 1 min. The test was performed three times at 0, 15, 30, and 60 min after administration of KST5468 and vehicle (5% DMSO), respectively.

2.6. Hot plate test

The effect of KST5468 on nociceptive thermal pain was determined using a hot plate test (Eddy and Leimbach, 1953). Briefly, mice were habituated to a plexiglass cylinder for 20–30 min, and a hot plate was electrically heated to 53 °C (Ghelardini et al., 2002; Zelcer et al., 2005). The mice were then placed in the plexiglass cylinder (30 cm in diameter, 40 cm in height) and the latency time of licking or shaking the hindpaws or jumping off from the plate was measured. A maximum cut-off time chosen to avoid tissue damage was 30 s. After baseline behavior tests, mice were immediately administered with drugs and the test was performed at several time points (15, 30, 45, and 60 min). Each mouse was tested three times with a time interval between each test.

2.7. Neuropathic pain models

2.7.1. Chronic constriction injury (CCI) surgery

For assessing the antinociceptive effect of KST5468 on neuropathic pain, two kinds of animal models were used in this study as previously described (Bennett and Xie, 1988). For chronic constriction injury (CCI) surgery, mice were anesthetized with 3% isoflurane via inhalation with a nose cone and the common sciatic nerve of the left hind limb was exposed at mid-thigh level just proximal to its trifurcation. The nerve was freed and four ligatures of 6-0 chromic gut (W812, Ethicon Inc., Somerville, NJ) were tied loosely with an approximate spacing of 1 mm. Following the nerve ligation, the muscle and the skin were closed with 6-0 silk sutures. The mice had a quick recovery within 5–10 min. Antibiotics or analgesics were not administered after the surgery.

2.7.2. Spared nerve injury (SNI) surgery

SNI surgery was carried out under 3% isoflurane anesthesia via inhalation with a nose cone. In the surgery, the left hind limb was incised at mid-thigh level and the overlying muscles were retracted to expose the peripheral branches of the sciatic nerve (common peroneal, tibial and sural). Both the common peroneal and the tibial nerves were ligated with 6-0 silk (W802, Ethicon Inc.) and transected together, leaving the sural nerve carefully preserved (Decosterd and Woolf, 2000). The mice had a quick recovery within 5–10 min. Antibiotics or analgesics were not administered after the surgery.

2.7.3. Behavior test

To investigate the effects of mechanical pain threshold in each neuropathic pain model, von Frey tests were performed. Mice were habituated to transparent plexiglass boxes (5×10×5 cm) placed on an elevated metal mesh floor for 20–30 min. Next, a series of 10 von Frey monofilaments were applied to external site of the lateral plantar surface of the hindpaw (SNI surgery group) which are innervated by the sural nerve, and the whole plantar surface of the hindpaw (CCI surgery group). The results of this test were determined by paw withdrawal response in three of five repetitive stimuli. In negative response cases, the next incrementally stiffer monofilament was applied until a pain response was observed (Decosterd and Woolf, 2000; Tal and Bennett, 1994). Two weeks after surgery, baseline responses were assessed before KST5468 injection. After the injection, the von Frey test was reevaluated after 30, 60, 90 and 120 min. For the von Frey test, gram values for the filament force were used rather than the mN value, because the contact area was not uniform due to the elastic character of the plantar skin.

2.8. Immunohistochemistry

ICR mice were deeply anesthetized with 3% isoflurane via a nose cone approximately 2–3 min before perfusion, then transcardially perfused with 40–60 ml saline followed by 50–100 ml of cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The L4–L5 segments of the spinal cord were subsequently dissected, and they were processed for immunohistochemistry which was previously described (Ahn et al., 2006; Lin et al., 2007; Tsuda et al., 2008). The sections were incubated with primary antibodies specific for rabbit-anti-c-Fos (1:10,000, Calbiochem, San Diego, CA), rabbit-anti-CGRP (1:10,000, Sigma-Aldrich, Saint Louis, MO) or rabbit-anti-p-ERK (1:200, Santa Cruz Biotech, Santa Cruz, CA). To detect the staining of each antibody, the sections were treated with horse radish peroxidase (Thermo Scientific Lab Vision) and were incubated in a diaminobenzidine (DAB) solution kit (Thermo Scientific Lab Vision) for approximately 3 min. The stained sections were cover-slipped using Permount (Sigma-Aldrich). Antibody expression patterns were recorded and analyzed with Image Pro 6.2 software (Media Cybernetics Inc., Bethesda, MD). The area of c-fos, CGRP and p-ERK expression was counted in 2–3 sections per ICR mice in the laminae I–II layers of the ipsilateral L4–L5 spinal dorsal horn, and calculated from the ratio of antibody expression area per whole the laminae I–II layers.

2.9. Data analysis

All data are expressed as the mean ± the standard error of the means (S.E.M.). SPSS version 17.0 software (SPSS Korea, Seoul, Republic of Korea) was used to analyze behavior studies. Statistically significant differences were determined using one-way ANOVA with the Tukey–Kramer post-test for multiple comparisons and Duncan's t-test. The values of $P<0.05$ were regarded as statistically significant.

3. Results

3.1. Pharmacokinetics of KST5468 after intravenous and intraperitoneal administration to mice

The pharmacokinetic profile of KST5468 after intraperitoneal and intravenous administration to ICR mice is shown in Tables 1 and 2. The $AUC_{0-\infty}$ values of KST5468 are 109.7 and 19.92 μg min/ml in intraperitoneal and intravenous administration, respectively. Bioavailability was moderate with 18.16%. The mean B/P ratio (AUC_{brain}/AUC_{plasma}) was 9.72 after intravenous administration and 9.45 in intraperitoneal administration.

3.2. Effect of KST5468 on a formalin-induced inflammatory pain model

The formalin test was performed to test the hypothesis that KST5468 has antinociceptive effects on inflammatory pain. In the formalin test, pain responses such as licking of the left hindpaw were expressed in two phases. The first phase was 0 to 5 min after formalin injection and the second phase was 20 to 40 min after formalin injection. The control group produced a typical nociceptive response in both the first and second phases. The licking time of the first phase was reduced (56.42 ± 13.23 s) when the mice were treated with

Table 1
Plasma pharmacokinetic parameters after intravenous and intraperitoneal administration (10 mg/kg) of KST5468 to ICR mice ($n=3$ per time point).

Plasma	Intravenous	Intraperitoneal
$AUC_{0-\infty}$(μg min/ml)	109.7	19.92
AUC_{last} (μg min/ml)	104.1	16.90
Terminal half-life (min)	193.3	159.3
C_{max} (μg/ml)	–	0.2986
T_{max} (min)	–	5
CL (ml/min/kg)	91.20	–
MRT (min)	100.7	–
V_{ss} (ml/kg)	9185	–
F (%)	18.16	

Table 2
Brain pharmacokinetic parameters after intravenous and intraperitoneal administration (10 mg/kg) of KST5468 to ICR mice ($n=3$ per time point).

Brain	Intravenous	Intraperitoneal
$AUC_{0-\infty}$(μg min/g)	1065	188.3
AUC_{last} (μg min/g)	1056	174.8
Terminal half-life (min)	119.0	119.6
C_{max} (μg/g)	32.95	1.495
T_{max} (min)	5	15
AUC_{brain}/AUC_{plasma} (%)	971.7	945.4

10 mg/kg injection of KST5468 compared to the control group (102.25 ± 8.27 s, P = 0.006). However, injection of 1 or 5 mg/kg of KST5468 or gabapentin did not affect licking time compared to the control group in the first phase. In the second phase, licking time was significantly reduced by both gabapentin (P = 0.004) and KST5468 (P = 0.0007). In addition, the licking time was reduced by gabapentin injection of 100 mg/kg (121.71 ± 35.82 s) and KST5468 injection of 10 mg/kg (113.85 ± 14.38 s) compared to the control group (246.5 ± 21.05 s). However, 1 and 5 mg/kg of KST5468 did not significantly reduce licking time in the second phase (Fig. 2).

3.3. Antinociceptive effect of KST5468 on noxious thermal stimuli

A hot plate test was performed to evaluate the antinociceptive effect of KST5468 on noxious thermal stimuli. The basal withdrawal latencies of KST5468, tramadol and DMSO treated groups were 13.03 ± 0.56 s 13.41 ± 1.25 and 12.39 ± 0.91 s, respectively. As shown in Fig. 3, only at 30 min, the paw withdrawal latency was significantly increased in animals injected with KST5468 and tramadol compared to the control treatments. The paw withdrawal latency was not significantly different between KST5468 and tramadol group throughout the experiment.

3.4. Effect of KST 5468 on mechanical hypersensitivity in two neuropathic pain models

To confirm the hypothesis that KST5468 has an antinociceptive effect on neuropathic pain, we evaluated mechanical allodynia after intraperitoneal injection of KST5468 (10 mg/kg). Two weeks after CCI or SNI surgery, mechanical thresholds were decreased to below 0.1 g, confirming that neuropathic pain was induced. Following the development of neuropathic pain, mice were injected with KST5468 14 days after the surgeries (Fig. 4). Compared with the pre-injected test (0.011 ± 0.001 g in CCI, 0.017 ± 0.004 g in SNI), the mechanical threshold, assessed by the von Frey paw withdrawal threshold, was significantly different at 30, 60, and 90 min after the injection of the compound in both models (P = 0.034, 0.038, 0.015 respectively in CCI and P = 0.014, 0.029, 0.0005 respectively in SNI), suggesting the antinociceptive effect of KST5468 in neuropathic pain models. However, the mechanical threshold after KST 5468 injection was significantly different at 90 and 120 min in CCI models and 60 and 90 min in SNI model compared to gabapentin group (P = 0.007 and 0.018 respectively in CCI and P = 0.005 and 0.001 respectively in SNI). In sham-operated control, the mechanical threshold was significantly increased compared to gabapentin and KST5468 groups in both CCI and SNI models (data not shown).

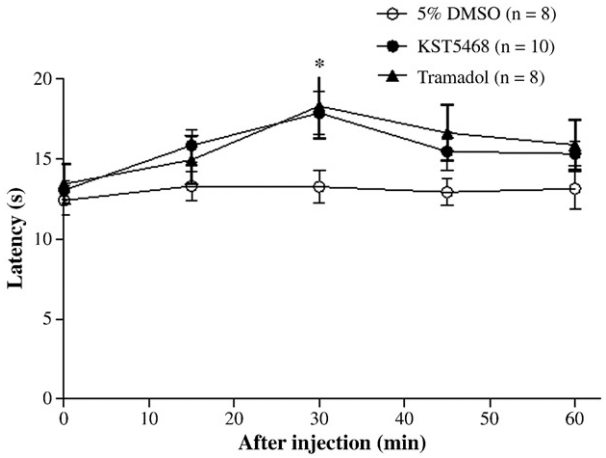

Fig. 3. The effect of KST5468 on noxious thermal stimuli. The mice were treated intraperitoneally with KST5468 (10 mg/kg), tramadol (30 mg/kg) or vehicle (5% DMSO). The withdrawal latency was measured in each group. *$P<0.05$ indicates a significant difference compared with the control group. Values are mean ± SEM ($n=8$–10).

To test the possibility that the KST5468-induced antinociceptive effect could be due to an alteration in the motor coordination, we performed the rotarod test. As shown in Fig. 5, no differences of the balance time on the rod between KST5468 treated and the control mice were observed. Thus, we believe that KST5468-induced behavioral changes may be solely due to the antinociceptive effect of KST5468.

3.5. Reduction of nociception-related induction of c-Fos, CGRP, and p-ERK in the spinal cord in KST5468 treated mice

Immunoreactivity analysis indicated the antinociceptive effect of KST5468 in the superficial layers (Rexed's laminae I–II) of the spinal dorsal horns at the molecular level. The cells positively expressed with each antibody at the superficial layers were counted and the area with positive expressing cells was calculated from dividing it by the total

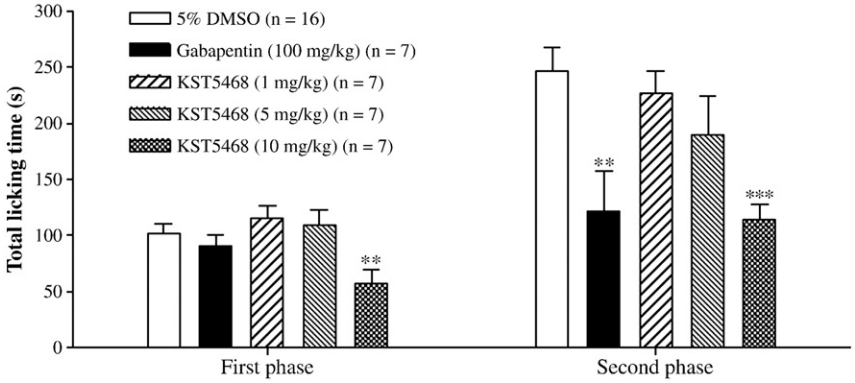

Fig. 2. The effect of KST5468 on formalin-induced inflammatory pain. The mice were treated with KST5468 (1, 5, 10 mg/kg i.p.), gabapentin (100 mg/kg, i.p.) or vehicle (5% DMSO). The tail licking time was measured in the first and second phases of the formalin test in each group. **$P<0.01$ and ***$P<0.001$ indicate a significant difference compared with the control group. Values are mean ± SEM ($n=7$–16).

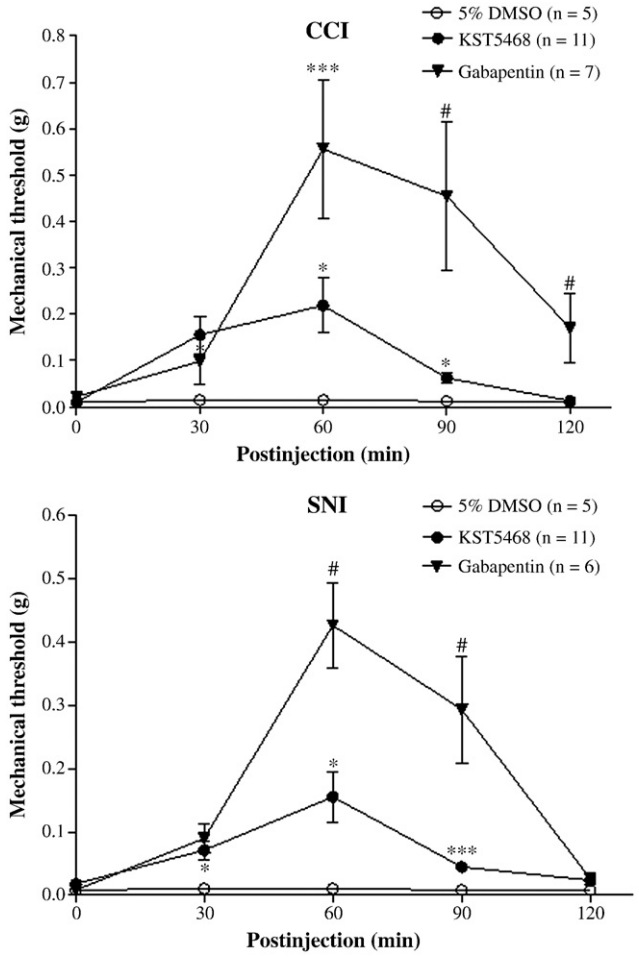

Fig. 4. The effect of KST5468 on mechanical hypersensitivity induced by chronic constriction injury (CCI) and spared nerve injury (SNI) in animal models of neuropathic pain. After the establishment of neuropathic pain models (14 days post-surgery; CCI and SNI models), the mice were treated intraperitoneally with KST5468 (10 mg/kg), gabapentin (100 mg/kg) or vehicle. The von Frey paw withdrawal threshold was measured in each group. $^{*}P<0.05$ and $^{***}P<0.001$ indicate a significant difference compared with the control group. #$P<0.05$ indicates a significant difference compared with KST5468 group. Values are mean ± SEM ($n = 5$–11).

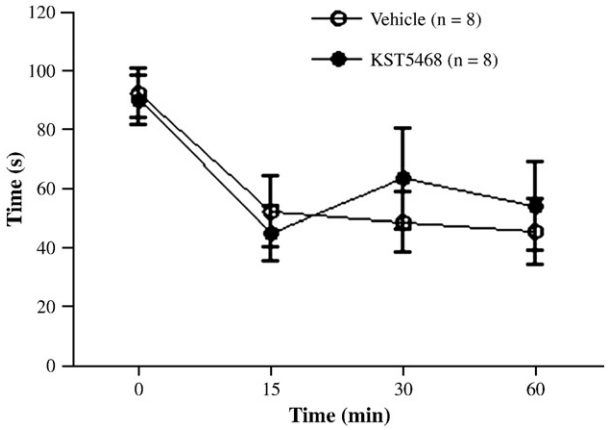

Fig. 5. The effect of KST5468 on motor coordination in mice. The mice preadministered intraperitoneally with KST5468 or vehicle (5% DMSO) were subject to the rotarod test at 0, 15, 30, and 60 min after injections. The time of maintaining balance on the rod was measured by a blinded observer for each group, respectively. The data was shown as mean ± SEM ($n = 8$ for KST5468 group and $n = 8$ for vehicle group).

cell counts as a percentage. The expression of c-Fos was significantly reduced in both models compared with the control ($P = 0.034$ in CCI, $P = 0.027$ in SNI). In addition, CGRP expression was similar to that of c-Fos, and significantly different in both models ($P = 0.024$ in CCI, $P = 0.034$ in SNI). Likewise, p-ERK expression was decreased in both models. Moreover, the statistical values of animals that underwent CCI surgery were significant ($P = 0.035$ in CCI, $P = 0.232$ in SNI) (Fig. 6).

4. Discussion

In this study, we showed that KST5468 alleviated pain behavior and significantly increased mechanical threshold of neuropathic pain as a result of decreased c-Fos, CGRP, and p-ERK expression in the spinal cord. Our results suggest that KST5468, a novel synthetic T-type Ca^{2+} channel antagonist, may be effective to treat neuropathic and inflammatory pain.

The hot plate test is usually used to assess analgesic effects against thermal pain. Based on our data, KST5468 may be efficacious on

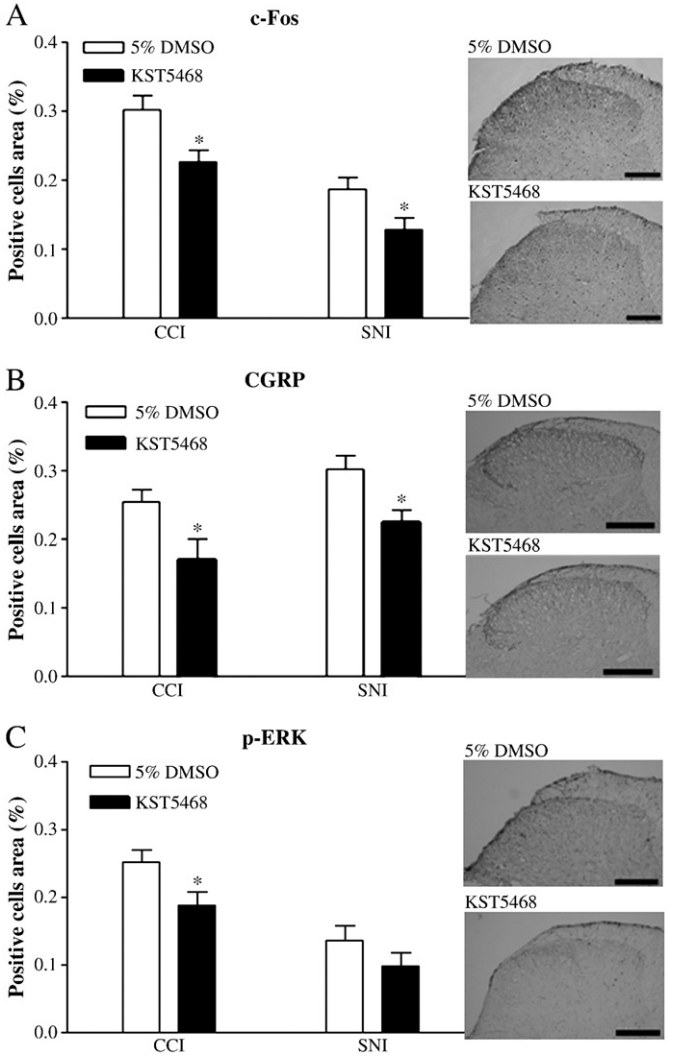

Fig. 6. Immunohistochemical analysis of the expression of c-Fos, CGRP, and p-ERK in the laminae I–II layers of the ipsilateral L4–L5 spinal dorsal horn in CCI and SNI neuropathic pain models. After the establishment of neuropathic pain model (SNI and CCI models) the mice were treated intraperitoneally with KST5468 (10 mg/kg) or vehicle. Expression levels of c-Fos, CGRP, and p-ERK expression were compared between the two groups. $^{*}P<0.05$ indicates a significant difference compared with the control group. All of the micrographics are chosen in CCI groups. Values are mean ± SEM. Scale bar = 200 μm.

thermal pain; however, this is controversial due to the presence of previous data indicating that the α1G subtype of T-type Ca^{2+} channels does not directly reduce spinal nociception by nociceptive thermal stimuli (Kim et al., 2003; Shin et al., 2008). Distinct from the α1G subtype, mice lacking α1H subtype of T-type Ca^{2+} channel have shown reduced pain behavior in acute thermal and mechanical pain stimuli (Choi et al., 2007). Our conflicting results may be due to the nonspecificity of KST5468 on calcium channel subtypes. We previously confirmed that KST5468 displayed an inhibitory effect on α1G subtype of T-type Ca^{2+} channels. However, the effect of KST5468 on other types of T-type Ca^{2+} channels has not been investigated yet. Until the effect of KST5468 on α1H subtype of T-type Ca^{2+} channel is clearly established, it will be difficult to understand this conflicting result. Also, the antinociceptive effect against thermal pain for a relative short duration (30 min) could be explained by relatively short time for KST5468 to reach maximum concentration in the plasma (5 min) and central nervous system (15 min, brain) and terminal half time although other pharmacokinetic factors may influence pharmacologic duration of KST5468.

Intraplantar injection of formalin produces biphasic nociception-related behavior, with the earliest (first) phase caused by acute nociceptive injury and the later (second) phase related to central sensitization (Dickenson and Sullivan, 1987). Inflammatory mediators such as bradykinin, histamine, and prostaglandin sensitize dorsal horn neurons by changing the ionic conductance (Ito et al., 2001). Previous research has shown that T-type Ca^{2+} channel α1G subtype KO mice exhibit increased nociceptive behaviors (Shin et al., 2008), whereas Cav2 KO mice showed a reduced response in the second phase of the formalin test (Choi et al., 2007). Additionally, T-type Ca^{2+} channel blockers including mibefradil and $NiCl_2$ reduce formalin-induced nociceptive behaviors (Cheng et al., 2007). The data generated in the present study is in agreement with the data from the previous study, and thus T-type Ca^{2+} antagonists may be a novel therapy for chronic inflammatory pain. Moreover, a novel T-type Ca^{2+} antagonist KST 5468, tested in this study, showed favorable pharmacokinetic profiles. The V_{SS} of KST5468 was considerably large (9185 ml/kg). This suggests that mouse tissues had good affinity to KST5468; this is supported by a high level of brain-to-plasma (B/P) ratio of KST5468. After intraperitoneal administration, the penetration of KST5468 to brain tissue was fast. This is indicated by the short period of time that KST5468 reached maximum generation in brain (15 min) and relatively high AUC_{brain}/AUC_{plasma} (9.45). Also, pharmacokinetic analysis suggests that KST 5468 is likely to act on T-type Ca^{2+} channel distributed in the central nervous system effectively.

In our study, KST5468 was not equivalent to gabapentin, which was used in neuropathic pain models as a positive control. Moreover, KST5468 did not restore von Frey threshold at peak effect to the level of sham-operated controls. This suggests that KST5468 may possess weaker effects in treating neuropathic pain compared to gabapentin. However, the pharmacokinetic feature of gabapentin is nearly identical to KST5468 (Radulovic et al., 1995). Thus, it is possible to speculate that pharmacodynamic differences may be the cause of the greater potency of gabapentin compared to KST 5468. Indeed, it is reported that gabapentin reduces pronociceptive neuron transmitter release by binding it with alpha 2 auxiliary subunit of N-type and/or P- and Q-type calcium channels (Fehrenbacher et al., 2003; Maneuf et al., 2004). Meanwhile, in our study, 10 mg/kg of KST5468 was found to have a similar pain-reducing effect on the second phase of the formalin test to 100 mg/kg of gabapentin. This suggests that the T-type Ca^{2+} channel blocker may be comparably effective in modulating central sensitization in comparison with gabapentin. Although KST5468 is less potent than gabapentin in treating neuropathic pain, it is encouraging that KST5468 is also effective in neuropathic pain as well as inflammatory and nociceptive pain.

Animal models such as the CCI and SNI models have been extensively used to study neuropathic pain behaviors and pharmacological responses. Both models closely mimic clinical features including the spontaneous pain and allodynia, which are frequently observed in neuropathic pain patients (Decosterd and Woolf, 2000), and mechanical allodynia has been regarded as an especially important phenomenon in neuropathic pain study (Mukhida et al., 2007; Truin et al., 2009). Several studies have been performed to investigate the effects of T-type Ca^{2+} channel blockers in various neuropathic pain models (Cheng and Chiou, 2006; Todorovic and Jevtovic-Todorovic, 2007). Consistent with previous studies, we found that intraperitoneal injection of KST5468, a new synthetic T-type Ca^{2+} channel antagonist, reduced mechanical hypersensitivity in both the CCI and SNI models.

At the tissue level, we observed the changes of expression levels of neuropathic pain-inducible c-Fos, CGRP, and p-ERK after KST5468 injection. Phosphorylated ERK is extensively expressed in the central nervous system and plays a pivotal role in signal transduction pathways regulating neuronal activity and plasticity. Recent studies have shown that ERK is also essential for pain sensation after peripheral stimulation and inflammation. For example, the protein expression of p-ERK is increased in the spinal dorsal horn by several reasons such as mechanical stimulation, noxious heat, and electrical stimulation of the peripheral nerve (Ji et al., 1999). Furthermore, the phosphorylation of ERK can control transcriptional regulation (Impey et al., 1999). Specifically, immediate early genes such as c-Fos induction are changed as a result of p-ERK expression patterns (Sgambato et al., 1998). Likewise, the expression of c-Fos in the spinal cord dorsal horn is regarded as a marker of neuronal activation after noxious stimulation (Herdegen and Leah, 1998). In addition to these indirect biological pain markers, CGRP is also widely distributed throughout the central and peripheral nervous systems and is known to contribute towards the generation and maintenance of pain sensations (Ju et al., 1987). As mentioned above, KST5468 increased the mechanical threshold in neuropathic pain models, and decreased p-ERK, c-Fos and CGRP protein expression in the laminae I–II layers of the ipsilateral L4–L5 spinal dorsal horn where the majority of small-unmyelinated C fibers and large-myelinated A_{β} fibers terminate. Thus, KST5468 reduced the reduction of neuropathic pain-induced by p-ERK, c-Fos, and CGRP expression; this suggests that the T-type Ca^{2+} channel antagonist may block the receptors of excitatory amino acid in the small size fibers terminating in the superficial laminae I–II layers of spinal dorsal horn.

In conclusion, we have identified KST5468 as a novel synthetic T-type Ca^{2+} channel antagonist that reduced pain-like behaviors in nociceptive, inflammatory and neuropathic pain animal models. Furthermore, we confirmed that KST5468 decreased c-Fos and CGRP expression in the superficial laminae of the L4–L5 spinal dorsal horn. Importantly, the findings of this study regarding KST5468 are consistent with previous reports regarding the antinociceptive effects of T-type Ca^{2+} channel blockers, and the findings suggest that the compound may be more effective than similar existing compounds. Thus, KST5468 may have therapeutic potential for clinical pain treatment, especially for neuropathic pain.

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