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AS1069562, the (+)-isomer of indeloxazine, but not duloxetine has a curative-like analgesic effect in a rat model of streptozotocin-induced diabetic neuropathy

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ABSTRACT

AS1069562 is the (+)-isomer of indeloxazine, which had been clinically used as a cerebral activator for the treatment of cerebrovascular diseases with serotonin and norepinephrine reuptake inhibition (SNRI) and neuroprotection. Here, we compared the analgesic effects of repeated treatment with AS1069562 and duloxetine, a selective SNRI, on pain-related behavior in a rat model of streptozotocin (STZ)-induced diabetic neuropathy. Further, we also evaluated the effects on the expression of neurotrophic factors and nerve conduction velocity. AS1069562 and duloxetine by single daily administration for 4 weeks significantly improved mechanical allodynia in STZ-induced diabetic rats and did not affect plasma glucose level or body weight. Interestingly, the analgesic effect of AS1069562 continued after a consecutive 1-week treatment discontinuation, although the plasma concentration of AS1069562 was reduced to undetectable levels. In contrast, the efficacy of duloxetine disappeared after treatment discontinuation. Expression analysis demonstrated that AS1069562 significantly restored decreased insulin-like growth factor 1 and fibroblast growth factor 2 mRNA levels in dorsal root ganglion and spinal cord, respectively, whereas duloxetine did not affect the expression levels of neurotrophic factors. In addition, AS1069562 reversed the slowing of nerve conduction velocity. The results of this study indicate that the analgesic effect of repeated dosing of AS1069562 but not duloxetine is persistent even after a 1-week drug discontinuation in STZ-induced diabetic rats. Restoration of neurotrophic factors may be involved in the curative-like pharmacological effect of this agent. Thus, AS1069562 may potentially offer a better treatment option for patients with painful diabetic neuropathy than duloxetine via different mechanisms.

1. Introduction

Diabetic neuropathy is one of the most common complications of diabetes mellitus. Patients with this neuropathy experience symptoms such as spontaneous pain, allodynia, and hyperalgesia with an incidence rate of 10–20% (Dyck et al., 1993; Harati, 1996). Symptoms of painful diabetic neuropathy are highly unpleasant for affected individuals and usually disruptive to daily life.

Serotonin (5-HT) and norepinephrine (NE) are implicated in the modulation of endogenous analgesic mechanisms via the descending inhibitory pain pathways in the central nervous system. 5-HT and NE reuptake inhibitors (SNRIs) are thought to exert an analgesic effect by enhancing the serotonergic and noradrenergic descending inhibition system. Antidepressants such as duloxetine, an SNRI, are often used for the treatment of neuropathic pain in patients with painful diabetic neuropathy. Although the clinical effects of SNRIs have been well established, their beneficial effects are based on transient symptomatic relief of pain, and they cannot cure the underlying condition itself. Thus, there are critical unmet needs for new drugs which exert a long-term analgesic effect on painful diabetic neuropathy.

Indeloxazine is an SNRI with potential antidepressant properties. This drug had been used for the treatment of psychiatric

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symptoms associated with cerebrovascular disease, namely post-stroke depression, emotional disturbance, and reduced volition, in Japan and South Korea (Yamamoto, 1990). In experimental rodent studies, indeloxazine has shown multiple pharmacological actions, including 5-HT and NE reuptake inhibition, facilitation of acetylcholine release in the frontal cortex, and neuroprotective effects after ischemic brain injury (Yamaguchi et al., 1997). AS1069562 is the optical (+)-isomer separated from a racemic compound, indeloxazine. Compared to indeloxazine, AS1069562 was approximately 25 times less potent in inhibiting NE uptake and about 3 times less potent in central arousal activity in rodents. On the other hand, AS1069562 was equipotent to indeloxazine in inhibiting 5-HT uptake and in affecting learning behavior in rodents (Shimizu-sasamata et al., 1993).

Streptozotocin (STZ), which selectively destroys pancreatic β cells (Rakieten et al., 1963), rapidly induces diabetes in rodents in a model of insulin-dependent diabetes mellitus. Prolonged hyperglycemia, hypoinsulinemia, and abnormal glucose metabolism cause the degeneration of neurons (Courteix et al., 1993). Decreases in neurotrophic factors occur in STZ-induced diabetic animals, as in patients with diabetic neuropathy, and this in turn exacerbates the degeneration of neurons (Craner et al., 2002; Guo et al., 1999). Slowing of nerve conduction velocity, a conventional electrophysiological parameter that reflects the severity of nerve dysfunction, also occurs in STZ-induced diabetic animals as in patients with diabetic neuropathy (Calcutt et al., 1996; Sharma and Thomas, 1974). In addition, STZ-induced diabetic rats show neuropathic pain, characterized by mechanical and thermal allodynia, one of the symptoms of diabetic neuropathy (Calcutt et al., 1996). Gabapentin, pregabalin, amitriptyline, mexiletine, and morphine, but not diclofenac, inhibit allodynia in a rodent model of STZ-induced diabetic neuropathy (Courteix et al., 1994; Field et al., 1999; Kiguchi et al., 2004; Ulugol et al., 2002; Yamamoto et al., 2009), suggesting that the STZ model is suitable for evaluating the clinical potential of compounds for treating painful diabetic neuropathy.

Here, we compared the analgesic effects of repeated treatment with AS1069562 and duloxetine on pain-related behavior in a rat model of diabetic neuropathy. Results showed that AS1069562 has a curative-like analgesic effect. The effects of AS1069562 and duloxetine on expression levels of neurotrophic factors in the dorsal root ganglion (DRG) and spinal cord as well as on nerve conduction velocity in the sciatic nerve were investigated after treatment discontinuation.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley (SD) rats (Charles River Laboratories Japan; Yokohama, Japan) were housed in polycarbonate cages with clean paper chips as bedding under controlled lighting (12-h light/dark cycle, lights on at 7:30 a.m.) and temperature (23 ± 1 °C). Rats had free access to water and food throughout the experiment. All animal experimental procedures were approved by the Animal Experiment Committee of Astellas Pharma Inc. All efforts were made to minimize the number of animals used and their suffering.

2.2. Induction of diabetes

Diabetic mellitus was induced via a single intravenous injection of STZ (Sigma, St. Louis, MO, USA) dissolved in pH 4.5 citrate buffer into the tail vein at a dose of 45 mg/kg. Age-matched control animals were injected with vehicle only. Animals were fasted from the evening prior to the day of STZ administration and were allowed to feed again after administration. Two weeks after STZ injection, plasma glucose levels were measured to examine the development of hyperglycemia, and rats with plasma glucose levels below 300 mg/dl were excluded from the study.

2.3. Measurement of plasma glucose levels

Blood samples were collected via the tail vein at 2 and 7 weeks after STZ injection. Plasma glucose levels were measured using the mutarotase-GOD method (Glucose C2; Wako Pure Chemical Industries, Osaka, Japan).

2.4. Drug administration

(R)-2-[(1H-inden-7-yloxy)methyl]morpholine monobenzenesulfonate (AS1069562) and duloxetine were synthesized at Astellas Pharma Inc. (Tokyo, Japan). Hydrochloride salts of AS1069562 and duloxetine were used as AS1069562 and duloxetine, respectively. For in vivo studies, AS1069562 and duloxetine were suspended in distilled water and administered orally at a volume of 5 ml/kg. Drug concentrations were calculated in terms of the free base. AS1069562 and duloxetine were administered at doses of 3, 10, and 30 mg/kg. At the doses AS1069562 and duloxetine enhanced 5-HT and NE transmission resulting from blockade of 5-HT and NE reuptake sites in rodent brain (Shimizu-sasamata et al., 1993; Koch et al., 2003). Administrations of AS1069562 and duloxetine were initiated at 3 weeks after STZ injection, and treatment was maintained daily for 4 weeks. The measurement of mechanical allodynia was performed at 1 and 3 h after first and last dosing of AS1069562 and duloxetine, respectively, representing time points with approximately maximum plasma concentration levels of the tested agents.

2.5. Pharmacokinetic study

Plasma samples were collected at 1 h after AS1069562 administration of 30 mg/kg in STZ-treated rats. At the same time points as used in mechanical allodynia measurements, plasma samples were collected after 4-week chronic administration of AS1069562 at 30 mg/kg and a consecutive 1-week treatment discontinuation. Three rats were used in each group. Samples were stored at −20 °C until use. Plasma samples were treated by protein precipitation with acetonitrile. After centrifugation (1500 g, 10 min, 4 °C), the supernatant was separated and used for analysis. The concentration of AS1069562 in the samples was quantified using high-performance liquid chromatography (Shimadzu Corp., Kyoto, Japan) coupled with a triple quadrupole mass spectrometer (API4000, Applied Biosystems, Rockville, MD, USA).

2.6. Measurement of mechanical allodynia (von Frey test)

Measurements of mechanical allodynia in STZ-injected rats were performed as described previously (Yamamoto et al., 2009). Rats were placed in acrylic cages with a wire grid floor and allowed to sit in a quiet room for 20–40 min prior to the start of tests. Before paw stimulation, the animals were quiet, but not lying on their paws, and had ceased to explore their cages or to mark them by defecating or urinating. An electronic von Frey system, which consists of a hand-held force transducer fitted with a 0.7 mm^2 polypropylene tip (electronic von Frey anesthesiometer, IITC Life Science Inc., Woodland Hills, CA, USA) was used. The tip was placed perpendicularly against the plantar surface of the hind paw with gradually increasing pressure. Actions such as vocalization, agitation, jumping, and avoidance were considered to be indicative of a withdrawal threshold. Voluntary movement associated with locomotion was not considered to be a withdrawal response. The force required to elicit a withdrawal response was recorded twice for each hind paw with an interval of at least 3 min. The mean of the four values was considered the withdrawal threshold (g) for each rat. Three weeks after STZ injection, the withdrawal threshold was measured for each rat, and rats were randomly assigned to experimental groups (control, vehicle-treated STZ, and AS1069562 or duloxetine at 3, 10, or 30 mg/kg-treated STZ) in such a way as to minimize the differences among group averages. Analgesic studies were performed after a single and 4-week chronic administration of drugs and after 1-week treatment discontinuation. Thirteen or fourteen rats were used in each group. All behavioral responses were measured by a researcher who was blinded to treatment group.

2.7. Gene expression analysis

Changes in gene expression were analyzed by quantitative polymerase chain reaction (PCR) analysis using the ABI PRISM 7900 sequence detection system (Applied Biosystems, Foster City, CA, USA) with SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. Briefly, DRGs and spinal cord from the L4-L6 region were isolated from control, vehicle-treated STZ, AS1069562-treated STZ, and duloxetine-treated STZ rats after 1-week treatment discontinuation of drugs at 30 mg/kg. Samples were obtained from three to six rats in each group, and total RNA was prepared with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and an RNeasy kit (Qiagen N.V., Venlo, the Netherlands) according to the manufacturer's instructions. Purified total RNA was reverse-transcribed to complementary DNA (cDNA) using Superscript III reverse transcriptase (Invitrogen) with random primers (Invitrogen). The cDNA was subjected to quantitative PCR analysis. Primers used to detect the messenger RNA (mRNA) for each gene are listed in Table 1. Gene expression was normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.8. Measurement of motor nerve conduction velocity (MNCV)

MNCV was measured with a Synax 1200 (NEC Medical Systems, Tokyo, Japan) by modification of a previously reported method (Cameron et al., 1989). Rats were anesthetized with pentobarbital, and rectal temperature was maintained at 37 ± 1 °C with a heating blanket. The sciatic nerve was exposed and electrophysiological recordings were carried out by placing the recording electrode around the gastrocnemius muscle of the foot and the stimulating electrode close to the sciatic

Table 1
Oligonucleotide primers used for quantitative PCR analysis of gene expression. Abbreviations: FGF1, fibroblast growth factor 1; FGF2, fibroblast growth factor 2; IGF1, insulin-like growth factor 1; NGFβ, nerve growth factor β; BDNF, brain-derived neurotrophic factor; S100β, S100 calcium binding protein B; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Gene	Gene symbol	RefSeqID	Forward primer	Reverse primer
FGF1	Fgf1	NM_012846	5′-TGTTCCCTTGACCATTGGCT-3′	5′-AATTCAGGCTCTGTGGGCTG-3′
FGF2	Fgf2	NM_019305	5′-GAGAAGAGCGACCCACACGT-3′	5′-TCCTCTCTCTTCTGCTTGGAGC-3′
IGF1	Igf1	NM_178866	5′-GCTGGTGGACGCTCTTCAGT-3′	5′-TTGAAGTAAAAGCCCCTTGGTC-3′
NGFβ	Ngf	XM_227525	5′-GATCGGCGTACAGGCAGAAC-3′	5′-TCTCCCTCTGGGACATTGCT-3′
BDNF	Bdnf	NM_012513	5′-AGGCACTGGAACTCGCAATG-3′	5′-AAGGGCCCGAACATACGATT-3′
S100β	S100b	NM_013191	5′-AGTGATGGAGACGCTGGACG-3′	5′-TCCTGGAAGTCACACTCCCC-3′
GAPDH	Gapdh	NM_017008	5′-CTTCTTGTGCAGTGCCAGCC-3′	5′-CACCGACCTTCACCATCTTGT-3′

nerve at the sciatic notch or knee. Stimulation with rectangular pulses (0.1 ms, 1 Hz) was delivered with supramaximal current, and 10 consecutive traces were recorded. MNCV was calculated by dividing the distance between the two stimulating electrodes by the averaged latency differences between the onset of M-waves evoked from the two sites. MNCV was evaluated in control, vehicle-treated STZ, and AS1069562 at 30 mg/kg-treated STZ rats after 1-week discontinuation of treatment. Ten or twelve rats were used in each group.

2.9. Experimental protocol

To evaluate the clinical potential of drugs for treating painful diabetic neuropathy, STZ-induced diabetic rats were prepared by STZ injection. Two weeks after STZ injection, plasma glucose levels were measured to examine the development of hyperglycemia. Three weeks after STZ injection, administrations of drugs were initiated, and treatment was maintained daily for 4 weeks to evaluate the effects of single and repeated treatment of drugs. After a single administration of drugs, mechanical allodynia was measured. After 4-week chronic administration of drugs, plasma glucose levels, body weights and mechanical allodynia were measured. After 1-week treatment discontinuation, measurements of mechanical allodynia, gene expression analysis, and measurement of MNCV were performed.

2.10. Statistical analysis

Data were expressed as the mean ± S.E.M. Significance of differences between two groups was assessed using Student's *t* test, while differences among more than two groups were assessed using Dunnett's multiple comparison test. $p < 0.05$ was considered significant.

3. Results

3.1. Effects of single and chronic administration of AS1069562 and duloxetine on mechanical allodynia in STZ-induced diabetic rats

Diabetic neuropathy was induced by administration of STZ in rats. STZ at 45 mg/kg significantly decreased the withdrawal threshold in response to stimuli to the hind paw with an electric von Frey system from 3 to 7 weeks after injection, indicating the animals had persistent mechanical allodynia, a symptom of neuropathic pain in STZ-induced diabetic neuropathy. Single daily oral administration of AS1069562 and duloxetine for 4 weeks was initiated at 3 weeks after STZ injection. Both AS1069562 and duloxetine induced significant recovery in withdrawal threshold after the first dosing at 30 mg/kg (Fig. 1A and B) and after the last dosing at over 10 mg/kg (Fig. 2A and B). Furthermore, the von Frey test was also performed after a 1-week drug washout to investigate the persistence of the analgesic effects of these agents. Interestingly, the analgesic effect of AS1069562 continued after consecutive 1-week treatment discontinuation, whereas that of duloxetine completely disappeared. Significant recovery of withdrawal threshold was maintained at over 10 mg/kg of AS1069562 (Fig. 3A and B). These results demonstrated that AS1069562 has a longer-lasting analgesic effect than duloxetine when administered for 4 weeks in a rat model of STZ-induced diabetic neuropathic pain.

3.2. Effects of chronic administration of AS1069562 and duloxetine on plasma glucose level and body weight in STZ-induced diabetic rats

To exclude the possibility that the analgesic effects described above might be confounded by hypoglycemic effects and global amelioration in diabetic condition caused by the drugs, we examined their effects on plasma glucose level and body weight after 4-week chronic administration. Results showed no significant differences between the vehicle-treated and AS1069562- or duloxetine-treated groups in plasma glucose level (Fig. 4) or body weight (Fig. 5).

3.3. Effects of chronic administration of AS1069562 on plasma concentration of AS1069562 in STZ-induced diabetic rats

Measurements of plasma concentrations of AS1069562 were performed 1 day as well as 4 weeks after the start of drug administration and also after the consecutive 1-week washout in STZ-induced diabetic rats. Plasma concentrations of AS1069562 at

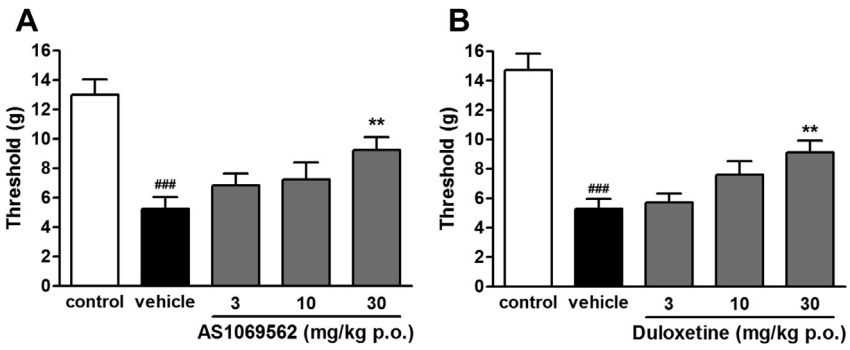

Fig. 1. Analgesic effects of AS1069562 (A) and duloxetine (B) after single oral administration on mechanical allodynia in a rat model of STZ-induced diabetic neuropathy. AS1069562 and duloxetine were orally administered 1 h and 3 h, respectively, before the von Frey test. Data are presented as the mean ± S.E.M. for 13 or 14 rats. ###, $p < 0.001$ by Student's *t* test compared with the control group. **, $p < 0.01$ by Dunnett's test compared with the vehicle-treated group.

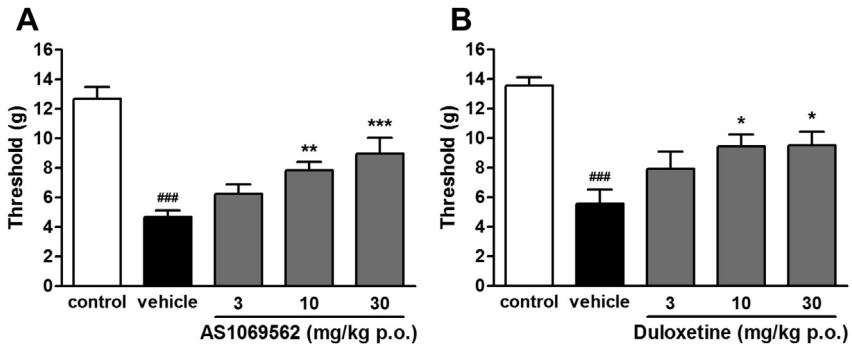

Fig. 2. Analgesic effects of AS1069562 (A) and duloxetine (B) after single daily oral chronic administration for 4 weeks on mechanical allodynia in a rat model of STZ-induced diabetic neuropathy. AS1069562 and duloxetine were orally administered 1 h and 3 h, respectively, before the von Frey test. Data are presented as the mean ± S.E.M. for 13 or 14 rats. ###, $p < 0.001$ by Student's t test compared with the control group. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ by Dunnett's test compared with the vehicle-treated group.

1 h after oral administration of 30 mg/kg on day 1 and after 4 weeks were 204 and 177 ng/ml, respectively, in STZ-treated rats. There was no significant difference in plasma concentration between single and 4-week administration. Plasma levels of AS1069562 were not detectable after the consecutive 1-week washout. The prolonged analgesic effect of AS1069562 observed after the 1-week drug washout was therefore not dependent on the continued presence of the drug in the plasma.

3.4. Effects of chronic administration of AS1069562 and duloxetine on expression levels of neurotrophic factors in STZ-induced diabetic rats

To determine the effects of AS1069562 and duloxetine on expression levels of neurotrophic factors in DRG and spinal cord, we performed quantitative PCR analysis of tissue samples from rats subjected to 4-week administration of AS1069562 and duloxetine at 30 mg/kg and a consecutive 1-week drug washout period. In order to investigate the different mechanism of AS1069562 from that of duloxetine to exert a curative-like analgesic effect, we selected the dose of 30 mg/kg, at which dose AS1069562 and duloxetine exerted different effects. DRG and spinal cord samples were collected from the L4, L5, and L6 levels because these levels receive input from the hind paw footpad, in which mechanical allodynia was measured. The results of quantitative PCR analysis are shown in Table 2. In L4, L5, and L6 DRGs from vehicle-treated STZ-induced diabetic rats, mRNA levels of fibroblast growth factor 1 and 2 (FGF1 and FGF2), insulin-like growth factor 1 (IGF1), and brain-derived neurotrophic factor (BDNF) were significantly reduced compared with normal control rats. In spinal cord from the L4-L6 region, mRNA level of FGF2 was significantly reduced compared with normal control rats. The expression of IGF1 in the DRGs and of FGF2 in the spinal cord was significantly restored by oral administration of AS1069562 at 30 mg/kg. It is notable that the level of FGF2 in spinal cord was completely recovered to the control level. In contrast, oral administration of duloxetine at 30 mg/kg did not affect the expression levels of neurotrophic factors in DRG or spinal cord.

3.5. Effects of chronic administration of AS1069562 on MNCV in STZ-induced diabetic rats

To investigate the effects of AS1069562 on nerve function, we measured MNCV of rats subjected to 4-week administration of AS1069562 at 30 mg/kg and a 1-week treatment discontinuation. We selected the dose of 30 mg/kg, at which dose AS1069562 showed significant analgesic effect after consecutive 1-week treatment discontinuation, in order to investigate the involvement of nerve function in a curative-like analgesic effect of AS1069562. MNCV of STZ-treated rats was significantly slowed as compared with control rats. Single daily chronic administration of AS1069562 at 30 mg/kg for four weeks induced a partial, but significant, reversal in the motor nerve conduction impairment in STZ-treated rats (Fig. 6).

4. Discussion

Treatment of painful diabetic neuropathy represents a major unmet medical need. Although a variety of pain management techniques are available, current treatments merely provide

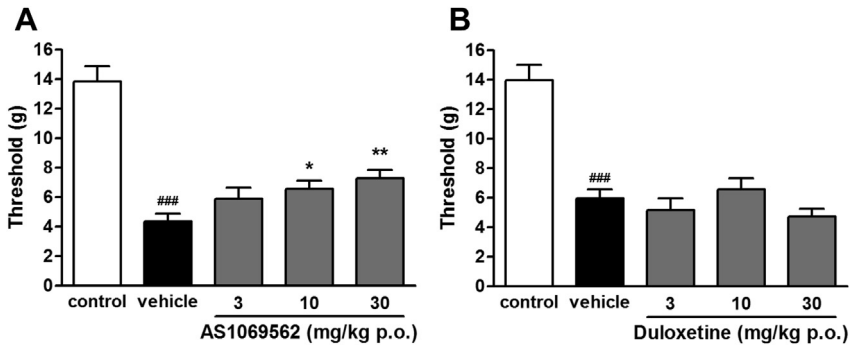

Fig. 3. Analgesic effects of AS1069562 (A) and duloxetine (B) after a 1-week treatment discontinuation on mechanical allodynia in a rat model of STZ-induced diabetic neuropathy. Data are presented as the mean ± S.E.M. for 13 or 14 rats. ###, $p < 0.001$ by Student's t test compared with the control group. *, $p < 0.05$; and **, $p < 0.01$ by Dunnett's test compared with the vehicle-treated group.

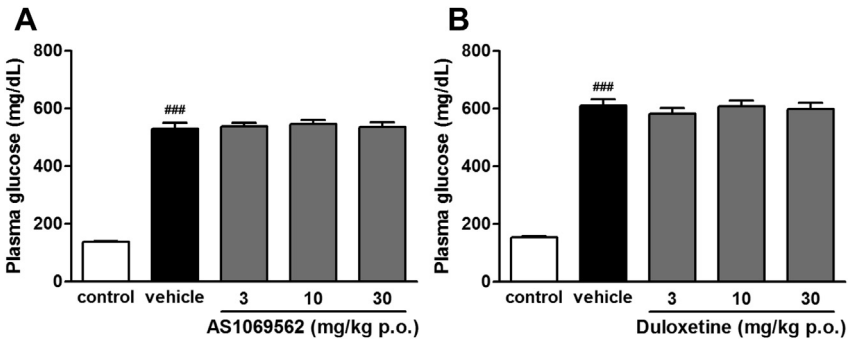

Fig. 4. Plasma glucose levels after single daily oral chronic administration of AS1069562 (A) and duloxetine (B) for 4 weeks in a rat model of STZ-induced diabetic neuropathy. AS1069562 and duloxetine were orally administered 1 h and 3 h, respectively, before sampling blood. Data are presented as the mean ± S.E.M. for 13 or 14 rats. ###, $p < 0.001$ by Student's *t* test compared with the control group.

symptomatic relief from pain without any effect on the underlying causes. Developing a drug that provides not only a symptomatic relief from pain but also a curative-like analgesic effect would meet the significant unmet medical needs of painful diabetic neuropathy.

The present study demonstrated the analgesic effects of single and chronic administration of AS1069562 in a rat model of STZ-induced diabetic neuropathy. In this model, single administration of AS1069562 improved mechanical allodynia, indicating that AS1069562 has an acute analgesic effect. Further, chronic administration of AS1069562 ameliorated mechanical allodynia. It is notable that pain improvement was observed up to one week after treatment discontinuation, at a time when plasma levels of AS1069562 were no longer detectable. Together, these findings suggest that AS1069562 provides not only symptomatic relief of pain but also a curative-like analgesia. We also demonstrated that single and chronic administration of duloxetine, which is used as a standard analgesic agent for the treatment of painful diabetic neuropathy, showed significant anti-allodynic effects in our STZ model, in accordance with other studies (Kuhad et al., 2009; Mixcoatl-Zecuatl and Jolivalt, 2011), confirming the predictive validity of this diabetic neuropathy model. However, duloxetine did not produce a long-lasting efficacy following treatment discontinuation, indicating that duloxetine lacks a curative-like effect and suggesting that AS1069562 might act via mechanisms different from duloxetine. Importantly, AS1069562 and duloxetine treatment did not affect hyperglycemic condition or body weight in diabetic animals, showing that the effects of AS1069562 and duloxetine treatment on nociceptive thresholds in diabetic rats were not due to any normalizing effects on hyperglycemia and body weight loss, which are indexes of global amelioration of diabetic condition.

It has been documented that one possible mechanism of the deterioration of sensory neurons in painful diabetic neuropathy is the loss of neurotrophic support. Neurotrophic factors are proteins that promote the development, survival and maintenance of specific neuronal populations. They cause responsive neuronal populations to be more resistant to injury, enhance their physiologic activity, and might also stimulate regeneration (Leinninger et al., 2004). Several neurotrophic factors have been tested in animal models of diabetic neuropathy with variable success (Apfel, 2002; Calcutt et al., 2008). To clarify the mechanisms mediating the curative-like effect of AS1069562 in STZ-induced diabetic neuropathy, we investigated the expression levels of neurotrophic factors in DRG and spinal cord. Results showed that the expression level of IGF1 in DRGs of STZ-induced diabetic rats was significantly reduced, in accordance with a previous study (Craner et al., 2002), and significantly restored by chronic administration of AS1069562. In addition, the expression level of FGF2 in spinal cord of STZ-treated rats was significantly reduced and restored to the normal level by chronic administration of AS1069562. Several studies have implicated reduced levels of IGF1 and FGF2 in diabetic neuropathy. For example, intrathecal delivery of IGF-1 improved slowing of nerve conduction velocity, atrophy in myelinated axons, and loss of epidermal fiber density and length independently of hyperglycemia in STZ-induced diabetic rats (Brussee et al., 2004; Toth et al., 2006). In addition, subcutaneous infusion of IGF1 prevented progressive hyperalgesia and partially reversed impaired sensory nerve regeneration independently of hyperglycemia in STZ-induced diabetic rats (Morgado et al., 2011; Zhuang et al., 1996). Further supporting a role for IGF1 in diabetic neuropathy, the decrease in circulating levels of IGF1 in patients with diabetes is greater in those with neuropathy than in those without neuropathic

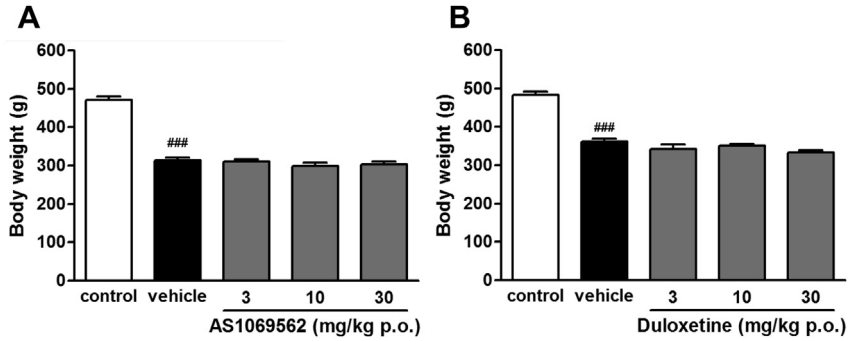

Fig. 5. Body weight after single daily oral chronic administration of AS1069562 (A) and duloxetine (B) for 4 weeks in a rat model of STZ-induced diabetic neuropathy. Data are presented as the mean ± S.E.M. for 13 or 14 rats. ###, $p < 0.001$ by Student's *t* test compared with the control group.

Table 2
Relative mRNA expression levels of neurotrophic factors in DRG and spinal cord from the L4-L6 level of STZ-induced diabetic rats after a 1-week treatment discontinuation of AS1069562 at 30 mg/kg p.o. or duloxetine at 30 mg/kg p.o. Values are normalized to the intensity of GAPDH and expressed as the mean ± S.E.M. of the percentage of the control level for 3 to 6 rats. #, $p < 0.05$; ##, $p < 0.01$; and ###, $p < 0.001$ by Student's t test compared with the control group.*, $p < 0.05$; and***, $p < 0.001$ by Student's t test compared with the vehicle-treated group.

Gene	Tissue	Control	STZ vehicle	STZ AS1069562 30 mg/kg, p.o.	STZ duloxetine 30 mg/kg, p.o.
FGF1	DRG	100 ± 9.3	65 ± 3.3##	76 ± 7.2	64 ± 8.0
	Spinal cord	100 ± 8.0	85 ± 2.8	78 ± 6.4	83 ± 8.1
FGF2	DRG	100 ± 4.8	23 ± 7.8###	21 ± 3.5	25 ± 4.5
	Spinal cord	100 ± 15	58 ± 4.4#	104 ± 4.9***	58 ± 6.1
IGF1	DRG	100 ± 14	55 ± 2.9#	68 ± 3.2*	45 ± 12
	Spinal cord	100 ± 8.0	67 ± 4.8	67 ± 2.7	67 ± 25
NGFβ	DRG	100 ± 29	107 ± 24	151 ± 41	100 ± 6.9
	Spinal cord	100 ± 13	110 ± 19	136 ± 18	119 ± 12
BDNF	DRG	100 ± 15	58 ± 2.6#	64 ± 3.0	49 ± 7.0
	Spinal cord	100 ± 6.9	82 ± 8.1	68 ± 7.0	80 ± 10
S100β	DRG	100 ± 20	55 ± 4.6	54 ± 6.3	50 ± 13
	Spinal cord	100 ± 7.0	98 ± 8.1	87 ± 5.2	92 ± 15

complaints (Guo et al., 1999; Migdalis et al., 1995). IGF1 exhibits glycemia-independent neuroprotective effects, preventing peripheral nerve damage and promoting nerve regeneration, which may account for the analgesic ability of IGF1 during painful diabetic neuropathy. In contrast, FGF2 administered intravenously or intramuscularly with cross-linked gelatin hydrogel improved delayed MNCV, hypoalgesia, and sciatic nerve blood flow in STZ-induced diabetic rats (Nakae et al., 2006). Furthermore, it has been reported that FGF2 has effects on the central nervous system (Abe and Saito, 2001; Katsuki et al., 2000) and peripheral nervous system (Fujimoto et al., 1997; Grothe and Nikkhah, 2001). Several lines of evidence suggest that FGF2 improves microvascular blood flow in the endoneurial microvasculature and promotes nerve regeneration, which might account for its ability to improve symptomatic diabetic neuropathy. With regard to other neurotrophic factors, AS1069562 treatment tended to increase the level of nerve growth factor (NGF) in DRGs in diabetic rats, although there were no differences between control and diabetic rats. In contrast, chronic administration of duloxetine did not affect expression levels of neurotrophic factors in DRG or spinal cord. Duloxetine did not exert restoration of IGF1 and FGF2 at a dose of 30 mg/kg, at which duloxetine inhibited 5-HT and NE reuptake and increased extracellular 5-HT and NE levels, suggesting that inhibition of 5-HT and NE transporters is not involved in the effect of AS1069562 on the two growth factors. It is well documented that chronic treatment with SNRIs increases BDNF levels in particular regions of the brain, such as the hippocampus (Calabrese et al., 2010; Molteni et al., 2009); however, no effects on BDNF levels in DRG or spinal cord were detectable after treatment with AS1069562 and duloxetine. Although further investigations are needed, the restoration of IGF1 and FGF2 expression levels suggests that AS1069562 might improve nerve function impairment via the amelioration of neurotrophic support.

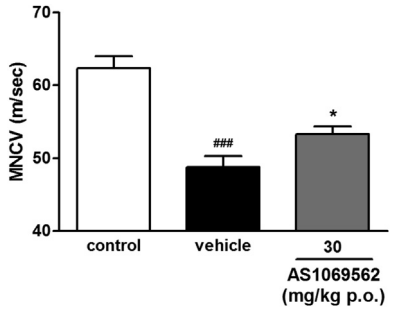

Fig. 6. Effects of AS1069562 after 1-week treatment discontinuation on MNCV in a rat model of STZ-induced diabetic neuropathy. Data are presented as the mean ± S.E.M. for 10 to 12 rats. ###, $p < 0.001$ by Student's t test compared with the control group. *, $p < 0.05$ by Student's t test compared with the vehicle-treated group.

In addition, the slowing of MNCV observed in STZ-induced diabetic rats (Sharma and Thomas, 1974), an endpoint used in diabetic neuropathy patients (Arezzo et al., 2008), was significantly improved by chronic administration of AS1069562. Several human and animal studies demonstrated that large myelinated (A-β) fibers as well as thinly myelinated (A-δ) fibers and unmyelinated (C) fibers are damaged in the diabetic neuropathic conditions (Dyck et al., 1976; Kapur, 2003). Nerve conduction velocity measurements are commonly used to assess nerve function in diabetes and exclusively reflect changes in large-diameter myelinated fibers (Arezzo and Zotova, 2002). Although the pain associated with painful diabetic neuropathy principally reflects dysfunction in small-diameter or unmyelinated (C) fibers, hypersensitivity to mechanical stimulation in diabetic rats persisted even when C fiber responses were abolished with resiniferatoxin, a potent analog of capsaicin (Khan et al., 2002), indicating that hypersensitivity to mechanical stimulation in diabetic rats is mediated by myelinated (A-β and A-δ) fibers rather than C-fibers. From this perspective, AS1069562 in the present study may likely have ameliorated mechanical allodynia and the decrease in nerve conduction velocity mainly via its improvement of damaged larger myelinated fibers in the diabetic neuropathic condition. Although the mechanism mediating the curative-like effectiveness of AS1069562 is not precisely known, improvement in nerve function via an improvement in the trophic support of neurons, which include larger myelinated fibers that mediate an abnormal painful response, may well be involved.

Currently, the target molecule of AS1069562 by which it elicits its curative-like effects remains unidentified. Previously, it was reported that indeloxazine produces a variety of pharmacological curative-like effects. In animal studies, indeloxazine showed anti-hypoxic and anti-ischemic actions due in part to the enhancement of energy metabolism. Indeloxazine ameliorated local cerebral glucose utilization in hypoxic rats (Miyaoka et al., 1988) and protected against cerebral damage resulting from an ischemic state in gerbils (Kano et al., 1993). Hypoxia is one of the main phenomena in diabetes associated with several complications, and it was demonstrated that hypoxia potentiates the activity of TRPV1, which acts as a detector and integrator of painful stimuli in sensory neurons (Ristoiu et al., 2011). This indicates that hypoxia might be one cause of pain in diabetic conditions, and that anti-hypoxic effects might be involved in an analgesic effect in diabetic conditions. In addition, indeloxazine increased the extracellular level of acetylcholine in rat brain (Yamamoto et al., 1993). A nicotinic acetylcholine receptor (nAChR) is involved in antinociception and neuroprotection as well as in cognitive function. As an example of this, the neuronal nAChR agonist ABT-594 exhibited analgesic activity in animal models of acute, chronic, and neuropathic pain (Kesingland et al., 2000). Furthermore, AS1069562 not only inhibits 5-HT and NE reuptake but also has an affinity to 5-HT_{1A} receptor (data not shown), and it was reported that high-efficacy 5-HT_{1A} receptor activation caused a curative-like action on allodynia in rats with spinal cord injury (Colpaert et al., 2004). Although the detail target mechanism has not been clarified, in addition to the restoration of expression levels of neurotrophic factors, other mechanisms such as anti-hypoxia, increase of acetylcholine release, and activation of 5-HT_{1A} receptor might contribute to the curative-like effect of AS1069562, which will be the future subjects to be clarified.

In summary, this study showed that chronic dosing of AS1069562, the (+)-isomer of indeloxazine, but not duloxetine caused persistent analgesia even after treatment discontinuation in a rat model of STZ-induced diabetic neuropathy. This curative-like analgesic effect of AS1069562 on painful diabetic neuropathy might be mediated via the restoration of IGF1 and FGF2 expression levels, resulting in the amelioration of nerve function impairment. These findings suggest that AS1069562 may represent a better treatment option for painful diabetic neuropathy than duloxetine via different mechanisms.

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