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Full paper

Analgesic effect of GT-0198, a structurally novel glycine transporter 2 inhibitor, in a mouse model of neuropathic pain

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ABSTRACT

This study was conducted to identify the characteristic pharmacological features of GT-0198 that is phenoxymethylbenzamide derivatives. GT-0198 inhibited the function of glycine transporter 2 (GlyT2) in human GlyT2-expressing HEK293 cells and did not bind various major transporters or receptors of neurotransmitters in a competitive manner. Thus, GT-0198 is considered to be a comparatively selective GlyT2 inhibitor. Intravenous, oral, and intrathecal injections of GT-0198 decreased the pain-related response in a model of neuropathic pain with partial sciatic nerve ligation. This result suggests that GT-0198 has an analgesic effect. The analgesic effect of GT-0198 was abolished by the intrathecal injection of strychnine, a glycine receptor antagonist. Therefore, GT-0198 is considered to exhibit its analgesic effect via the activation of a glycine receptor by glycine following presynaptic GlyT2 inhibition in the spinal cord. In summary, GT-0198 is a structurally novel GlyT2 inhibitor bearing a phenoxymethylbenzamide moiety with *in vivo* efficacy in behavioral models of neuropathic pain.

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1. Introduction

Glycine acts as an inhibitory neurotransmitter on the strychnine-sensitive glycine receptors (1), which are abundant in the spinal dorsal horn (2–4). In an animal model of neuropathic pain, chronic and single intrathecal administration of glycine suppressed mechanical hyperalgesia (5) and thermal hyperalgesia (6), respectively. These findings suggest that dysfunction of inhibitory synaptic transmission by glycine in the spinal dorsal horn is involved in the development of neuropathic pain (7). In fact, the glycine receptor antagonist strychnine elicits mechanical allodynia that is caused by glycinergic disinhibition in the spinal dorsal horn (8, 9). Thus, an enhancement of glycinergic inhibition in the spinal dorsal horn is a potential strategy for the relief of neuropathic pain.

The extracellular concentration of glycine is regulated by its reuptake into glycinergic presynaptic terminals and uptake into glial cells adjacent to inhibitory and excitatory synapses via Na^+/Cl^--dependent glycine transporters (GlyTs) (10). Two GlyT subtypes encoded by distinct genes, GlyT1 and GlyT2, have been identified in the mammalian central nervous system (1, 11). Recent studies have demonstrated that intrathecal injections of GlyT1 and GlyT2 inhibitors produce analgesic effects in animal models of neuropathic and inflammatory pain (12–14). The GlyT2 subtype is expressed in presynaptic glycinergic neurons of the spinal cord. Based on this localization, GlyT2 is believed to contribute to the clearance of glycine from the synapse at inhibitory synapses. On the other hand, GlyT1 is expressed in glial cells surrounding not only glycinergic but also glutaminergic synapses (1, 15). Therefore, GlyT1 inhibitor increases glycine concentrations at the glutaminergic terminal and may activate glutamate receptors, specifically N-methyl-D-aspartate (NMDA) receptors, given that glycine has been identified as a required coagonist with glutamate for NMDA receptors. Once an NMDA receptor is activated by the GlyT1 inhibitor, excitatory synaptic transmission is enhanced. These results indicate that a GlyT1 inhibitor has 2 effects: analgesic and algesic. Thus, GlyT2 inhibitors may be more useful than GlyT1 inhibitors as analgesic drugs.

Recently, we explored the structurally novel GlyT2 inhibitor by structural modifications of phenoxybenzamide derivative and we successfully designed GT-0198 that has strong potency of inhibition of GlyT2 (Fig.1). Furthermore, GT-0198 showed the good *in vivo* pharmacokinetics parameters (Table 1). Therefore, GT-0198 is expected to become a therapeutic agent of neuropathic pain by oral administration as tablets or capsules. In the present study, we show

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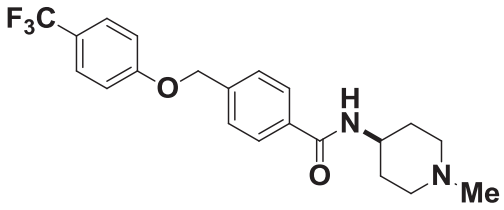

Fig. 1. The chemical structure of GT-0198.

the analgesic effect of GT-0198 in an animal model of neuropathic pain.

2. Materials and methods

2.1. Animals

Male ICR mice (5 weeks old at the start of experiments; Japan SLC Inc., Shizuoka, Japan) were used. All mice were housed 3–5 animals per cage under a room temperature of 21°C–25 °C, humidity of 40%–70%, and 12-h light/dark cycle (light on at 7:00 AM) for at least 1 week before use. Mice had *ad libitum* access to food and water. All experiments were conducted according to the Guidelines for Animal Experiments, Research & Development Division, Toray Industries, Inc., and a pain test was performed according to the Guidelines on Ethical Standards for Investigation of Experimental Pain in Animals (16).

2.2. Surgical operation

The mice were anesthetized with sodium pentobarbital (70 mg/kg, i.p.). We produced a partial sciatic nerve ligation (PSNL) model by tying a tight ligature with an 8–0 silk suture around approximately one-third to one-half the diameter of the sciatic nerve on the right side as described previously (17, 18). The sham-operated group was subjected to all procedures except ligation. In the present study, the pain test was performed 7 days after nerve ligation.

2.3. Pain test

To evaluate the analgesic effect of the compounds, paw withdrawal responses were measured using a von Frey filament (North Coast Medical, Morgan Hill, CA, USA) with a bending force of 0.16 g. The mice were placed individually in acrylic cages with wire mesh bottoms. After at least 60 min of acclimation, the von Frey filament was applied to the plantar surface of the hind paws for 3 s, and this was repeated 3 times. Each hind paw of the mice was tested individually. Paw withdrawal response to the von Frey filament was evaluated by scoring as follows: 0, no response; 1, slow and/or slight response to the stimulus; 2, quick withdrawal response away from the stimulus without flinching or licking; 3, intense withdrawal response away from the stimulus with brisk flinching and/or licking. The sum of 3 values served as the pain-related score.

Table 1
Pharmacokinetic parameters (brain and plasma ratio) of GT-0198 after oral administration (10 mg/kg).

Brain concentration (ng/g)	4478
Plasma concentration (ng/g)	300
Brain/Plasma ratio (%)	16.7

Blood and brain samples were collected at 1 h. Blood samples were centrifuged at 3000 g for 10 min and stored at −20 °C until analysis. Brain samples were homogenized and stored at −80 °C until analysis.

2.4. Glycine uptake assays with GlyT1 and GlyT2 transfected human embryonic kidney 293 cells

A vector expressing human GlyT1 or GlyT2 (GeneCopoeia, Inc. Rockville, MD, USA) was transfected into human embryonic kidney 293 (HEK 293) cells using Lipofectamine 2000 (Life Technologies Corporation, Carlsbad, CA, USA). The transfected HEK293 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum. Stable single-cell clones for hGlyT1 or hGlyT2 were isolated by selection with 500 μg/mL geneticin. Selected clones were evaluated in [^{3}H]-glycine uptake assays. HEK293 cells were plated (6000 cells per well) in polylysine-coated 96-well CytoStar-T® plates (Perkinelmer, Waltham, MA, USA) and incubated overnight. The cells were then incubated at 37 °C for 2 h with 240 nM [^{3}H]-glycine together with any compound being evaluated, in a total volume of 0.5 mL. At the end of the incubation, the radioactivity content of the wells was measured using Packard TopCount (Perkinelmer, Waltham, MA, USA).

2.5. Binding assays to various transporters and receptors of neurotransmitters

To evaluate the affinity of GT-0198 for various transporters and receptors, the rate of inhibition of the binding of a specific ligand to each transporter or receptor was assayed, as shown in Table 2. These assays were carried out in Sekisui Medical co., Ltd. Test substance concentration is 10 μM and positive substance concentration is 1 or 10 μM. Data are expressed as the mean values of duplicate samples. The inhibition ratios were calculated from "100 − binding ratio".

Binding ratio:

(Bound radioactivity in the presence of the test substance − Non-specific bound radioactivity)/(Total bound radioactivity in the absence of the test substance − Non-specific bound radioactivity) × 100 (%)

2.6. Drugs

GT-0198 was synthesized by Toray Industries Inc. Pregabalin was obtained from Bosch Scientific (New Brunswick, NJ, USA) and strychnine was obtained from Sigma (St. Louis, MO, USA). For

Table 2
Effect of GT-0198 on Radioligand binding to various transporters and receptors of neurotransmitters.

Target	Species	Inhibition (%)	
		GT-0198	Positive substance
Adenosine transporter	Human	4.24	100.00 (NBTI)
Monoamine transporter	Human	1.02	99.78 (Ketanserin)
Norepinephrine transporter	Human	11.02	100.00 (Desipramine)
Dopamine transporter	Human	23.16	99.70 (GBR12909)
GABA transporter	Rat	0.49	97.61 (GABA)
Serotonin transporter	Human	20.05	100.00 (Imipramine)
GABA A (Agonist site)	Rat	0.00	99.80 (Muscimol)
GABA A (BZ central)	Rat	0.02	100.00 (Diazepam)
GABA A (Chloride channel)	Rat	0.00	96.23 (Picrotoxin)
GABA B	Rat	2.00	100.00 (GABA)
Glutamate (AMPA)	Rat	2.29	100.00 ((S)-AMPA)
Glutamate (Kainate)	Rat	0.00	100.00 (Kainic acid)
Glutamate (NMDA agonist site)	Rat	1.71	99.23 (L-Glutamic acid)
Glutamate (NMDA glycine site)	Rat	0.00	99.49 (MDL105,519)
Glutamate (NMDA Phencyclidine site)	Rat	6.48	100.00 ((+)-MK-801)
Glutamate (NMDA polyamine site)	Rat	11.89	99.85 (Ifenprodil)
Glycine (Strychnine sensitive)	Rat	24.26	100.00 (Strychnine)

The concentration of GT-0198 is 1 μM. Data are expressed as the mean values of duplicate samples.

GABA = gamma-aminobutyric acid; NMDA = N-methyl-D-aspartate; AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; PCP = phencyclidine, NBTI = S-(4-Nitrobenzyl)-6-thioinosine.

intravenous or intrathecal injection, the compounds were dissolved in saline and injected in a volume of 0.1 mL/10 g body weight (intravenously) or 5 μL/body (intrathecally). For oral administration, the compounds were dissolved in distilled water and injected in a volume of 0.1 mL/10 g body weight.

2.7. Data analysis

Data are expressed as mean ± S.E.M. Difference among group for analgesic effects was determined using two-way analysis of variance (ANOVA) followed by suitable post-hoc test. $P < 0.05$ was considered to be significant.

3. Results

3.1. Inhibition of GlyT2 by GT-0198

GT-0198 inhibited the function of GlyT2 in human GlyT2-expressing HEK293 cells, with an IC_{50} value of 105 nM (Fig. 2). In contrast, GT-0198 did not suppress the function of GlyT1 (Fig. 2). Moreover, GT-0198 did not bind major transporters or receptors of neurotransmitters in the spinal cord in a competitive manner (Table 2). We accordingly inferred that GT-0198 is a compound that inhibits GlyT2 without inhibition of major transporters and receptors of neurotransmitters in the spinal cord.

3.1.1. Analgesic effects of systemic injection of GT-0198 on withdrawal responses in animal models of neuropathic pain

The effect of GT-0198 and pregabalin on withdrawal responses to mechanical stimuli was investigated in PSNL model mice (Fig.3). The model mice showed increased pain-related scores compared with sham-operated mice. Intravenous injection of GT-0198 decreased the pain-related score (Fig. 3A). Oral injection of GT-0198 also reduced the score in a dose-related manner, with statistical significance at ≥10 mg/kg (Fig. 3B). Pregabalin also significantly decreased the pain-related score at a dose of 10 mg/kg. In these experiments with GT-0198, no side effects possibly caused by glycine, such as convulsions or tremors, were observed.

3.1.2. Effect of intrathecal injection of GT-0198 on withdrawal responses in animal models of neuropathic pain

Because GlyT2 is abundant in the spinal dorsal horn, the analgesic effect of intrathecally injected GT-0198 was investigated in a mouse model of neuropathic pain. Intrathecal injection of GT-0198 decreased the pain-related score in a dose-related manner, with statistical significance at ≥1 μg/site (Fig. 4). Pregabalin also significantly decreased the pain-related score at a dose of 10 μg/site.

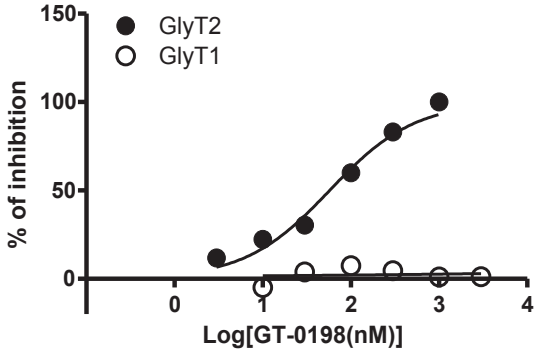

Fig. 2. Effect of GT-0198 on glycine uptake via GlyT1 or GlyT2 in transfected human embryonic kidney 293 cells. 100% inhibition means fully blocked by ORG24598 (3 μM) and ORG25543 (100 nM) in GlyT1 and GlyT2 assay, respectively. Data are mean value of representative experiments performed in duplicate.

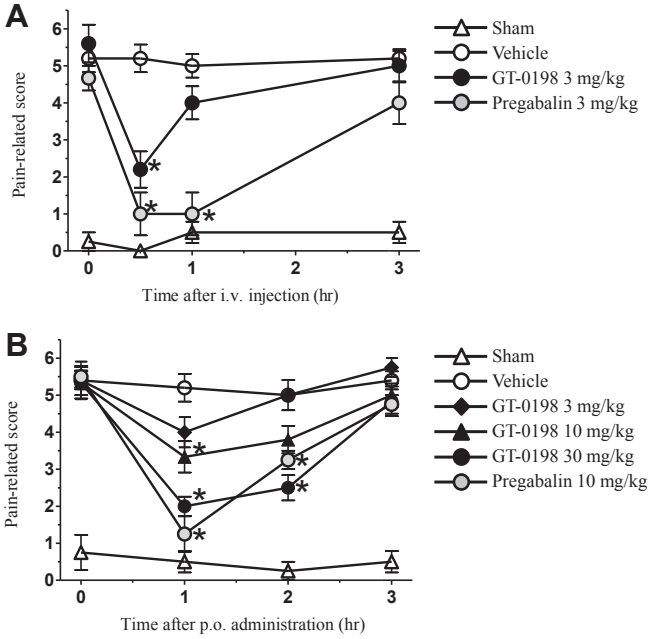

Fig. 3. Effects of GT-0198 on pain-related behavioral responses to mechanical stimuli in mice with partial sciatic nerve ligation. GT-0198, pregabalin, and vehicle were injected intravenously (A) and orally (B) 7 days after nerve ligation. The hind paws were stimulated using a von Frey filament with a bending force of 0.16 g. The values represent the mean and S.E.M. of 4–6 animals. *$P < 0.05$ vs. vehicle-injection (Two-way ANOVA).

3.2. Antagonistic effect of strychnine on the analgesic effect of GT-0198

To determine whether the action of GT-0198 was glycine- and glycine receptor-mediated following inhibition of GlyT2, the antagonistic effect of strychnine (a glycine receptor antagonist) on the analgesic effect of GT-0198 was evaluated. The analgesic effect of intravenous injection of GT-0198 was antagonized by intrathecally administered strychnine in all experimental periods (Fig. 5). Injection of strychnine alone in the spinal cord showed no effect on the pain-related score (data not shown).

4. Discussion

In this study, we investigated whether GT-0198, a structurally novel GlyT2 inhibitor that suppresses the uptake of glycine in human GlyT2-expressing HEK293 cells with submicromolar IC_{50}

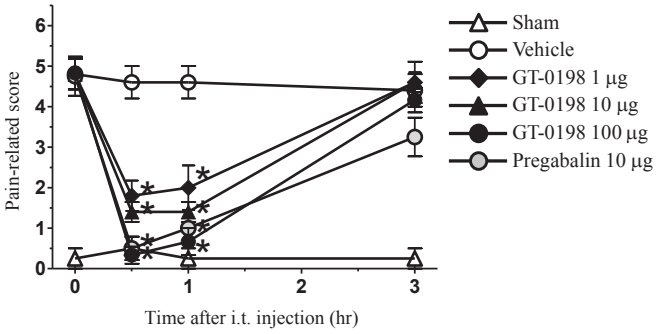

Fig. 4. Effects of GT-0198 on pain-related behavioral responses to mechanical stimuli in mice with partial sciatic nerve ligation. GT-0198, pregabalin, and vehicle were injected intrathecally 7 days after nerve ligation. The hind paws were stimulated using a von Frey filament with a bending force of 0.16 g. The values represent the mean and S.E.M. of 4–6 animals. *$P < 0.05$ vs. vehicle-injection (Two-way ANOVA).

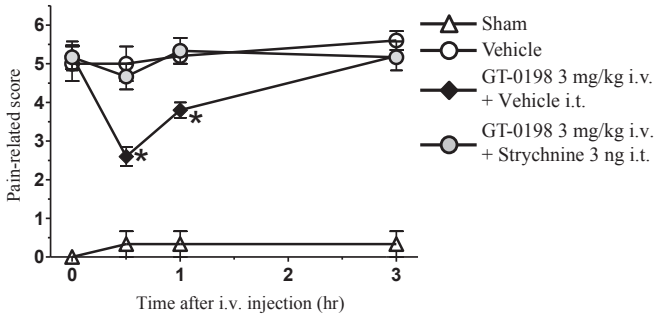

Fig. 5. Effects of strychnine on analgesic-like effect of GT-0198. GT-0198 was injected systemically; 15 min later, strychnine was injected intrathecally. At 0.5, 1, and 3 h after the injection, the hind paws were stimulated using a von Frey filament with a bending force of 0.16 g. The values represent the mean and S.E.M. of 3–6 animals. *P < 0.05 vs. vehicle-injection (Two-way ANOVA).

values, has a potent analgesic effect on a mouse model of neuropathic pain. Systemic (10 and 30 mg/kg; p.o.) and intrathecal (1, 10, and 100 μg/site) injection of GT-0198 exhibited an analgesic effect. The analgesic efficacy of GT-0198 was almost the same as that of pregabalin at a dose of 10 mg/kg by systemic injection or 100 μg/site by intrathecal injection. GT-0198 did not inhibit GlyT1 and did not bind to major receptors or transporters of neurotransmitters such as GABA, serotonin, and glutamate (Table 2). Thus, we considered that GT-0198 is a selective GlyT2 inhibitor and that its analgesic effect occurs at least partly via inhibition of glycine uptake in the spinal cord.

In previous studies, ALX1393 and ORG25543, typical GlyT2 inhibitors, showed analgesic effects against neuropathic pain and mechanically, heat-, and formalin-induced acute pain (13,19,20). However, these compounds showed minimal brain penetration. The brain/plasma ratio is 0.0036 and 0.53 after intravenous injection of ALX1393 and ORG25593, respectively (20). On the other hand, this ratio of GT-0198 is 16.7 after oral administration. Therefore, GT-0198 bearing a phenoxymethylbenzamide moiety has improved poor *in vivo* pharmacokinetics parameters property of two published GlyT2 inhibitors.

Because oral administration of GT-0198 showed analgesic properties, we propose that GT-0198 may be delivered as tablets or capsules. Moreover, GT-0198 produced no side effects such as convulsions, tremors, or even sedation or motor disinhibition. Thus, we expect GT-0198 to become an analgesic drug that can be used in clinical practice.

GlyT2 is restricted to glycinergic synapse-rich regions in the central nervous system, including the spinal cord (15, 21). Based on this localization, GlyT2 is believed to function as a glycine reuptake transporter at inhibitory glycinergic synapses (15, 22). In a previous study, glycinergic transmission was increased by the pharmacological blockade of GlyT2 in lamina X neurons of rat spinal cord slices (23). An increase in extracellular glycine concentrations was also demonstrated by microdialysis perfusion of the dorsal spinal cord of rats with the GlyT2 inhibitor ORG25543 (24). Taken together, these findings suggest that the analgesic effect of intrathecal GT-0198 observed in the present study results from the accumulation of glycine at the glycinergic synaptic cleft and subsequent suppression of excitatory neuronal activities in the spinal dorsal horn. In fact, glycinergic inhibitory postsynaptic currents were markedly reduced in the motor neurons from GlyT2-deficient mice and the analgesic effect of the systemic injection of GT-0198 was abolished by the intrathecal injection of the glycine receptor antagonist strychnine.

In this experiment, GT-0198, considered to be a GlyT2 inhibitor, showed an analgesic effect on a mouse model of neuropathic pain. The analgesic effect of a GlyT2 inhibitor has been shown in not only neuropathic pain but also another pain models. For example, N-arachidonylglycine, known as a GlyT2 inhibitor (25), was effective in suppressing phase 2 [acute inflammatory phase (26, 27)] of formalin-induced pain behaviors (28) and also in an animal model of complete Freund's adjuvant-induced inflammatory pain (13). These results suggest that GlyT2 inhibitors are suitable as a therapeutic agent even for inflammatory pain. Moreover, intrathecal injection of the GlyT2 inhibitor ALX1393 dose-dependently suppressed dynamic allodynia in mice with herpetic and postherpetic pain induced by percutaneous inoculation with herpes simplex virus (HSV) type 1 (29). This model shows zoster-like lesions throughout the inoculated dermatome (30–32), which may be caused by proliferation of HSV in the dorsal root ganglion (30, 33). These results suggest that GT-0198, considered to be a GlyT2 inhibitor, may alleviate pain symptoms that accompany neuropathic, inflammatory, herpetic, and postherpetic pain.

The GlyT2 inhibitor ALX-1393 significantly increased intercontraction interval and micturition pressure threshold in cyclophosphamide-treated rats (34). These results indicate that inhibition of GlyT2 leads to amelioration of cyclophosphamide-induced bladder overactivity and that GT-0198 may be a drug for the treatment of overactive bladder.

In summary, we have shown that GT-0198 had an analgesic effect in an animal model of neuropathic pain and suggest that GT-0198 could reduce this pain by inhibiting spinal GlyT2. GT-0198 is a structurally novel compound, with demonstrated analgesic efficacy in a behavioral model of neuropathic pain.

Conflicts of interest

The authors have no conflict of interest directly relevant to the content of this article.

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