GHS-R発現COS7細胞を用いて，六君子湯がグレリン受容体シグナルに及ぼす効果を解析した。GHS-R発現細胞に六君子湯（10～100μg/mℓ）を前処置（2～60分間）し，グレリンによるGHS-Rを介した細胞内$Ca^{2+}$濃度変化に及ぼす効果を解析した。

## Ⅲ 結果

① (1) 85As2細胞が及ぼす悪液質誘発メカニズムの解析：85As2細胞培養上清中のインターロイキン（IL）-1β，IL-6および腫瘍壊死因子（TNF）-αは検出限界以下であった。しかしIL-8および白血球遊走阻止因子（LIF）は10～1,000pg/mℓの範囲で有意に検出された。

(2) 悪液質発症後の腫瘍摘出が悪液質症状に及ぼす影響：85As2細胞移植による悪液質モデルラットは，腫瘍摘出により体重，摂食量，飲水量の低下，筋肉・脂肪組織重量低下などの悪液質症状がほぼ完全に回復し，また血中LIF濃度は検出限界以下となった。

(3) 筋肉分解因子の測定：悪液質モデルラットでは，筋肉分解に働くAtrogin-1/MAFbxおよびMuRF-1が対照（非担がん動物）群と比較して有意に増加していた。

(4) グレリン投与による摂食亢進作用：対照（非担がん動物）群では，グレリンにより有意な摂食量の増加が認められたが，悪液質モデルラットではグレリン投与による摂食量増加は認められなかった。

(5) 血中グレリン濃度：悪液質モデルラットは，対照（非担がん動物）群と比較して血中グレリン濃度が有意に高い値を示した。

② (1) 悪液質モデルラットを用いた六君子湯の悪液質改善への効果の検討：85As2細胞移植ラットでは，移植2週目より有意な体重減少および摂食量低下が認められ，特徴的な悪液質症状を示したのに対し，六君子湯予防投与群では体重には影響なかったが，移植4週目での有意な摂食量の改善を認めた。また悪液質を発症したモデルへの六君子湯7日間投与群では摂食量低下の有意な改善が認められ，さらに六君子湯は体重減少をも有意に抑制し，除脂肪量および体水分量も増加させ，加えて筋肉量も増加させた。しかし上昇している血中グレリン値のさらなる上昇は認められなかった。

(2) 六君子湯のGHS-Rシグナルに対する作用：細胞内カルシウム濃度アッセイにおいて，グレリン添加によりGHS-R安定発現COS細胞の細胞内$Ca^{2+}$濃度の上昇が認められ，六君子湯前処置によりグレリンによる細胞内$Ca^{2+}$濃度上昇がさらに増強されることが判明した。

## Ⅳ 考察

今回の実験結果より，ヒト胃がん細胞株由来の腹膜播種転移株85As2細胞を接種することにより作製したがん悪液質モデルラットは，体重減少，摂食量低下，除脂肪量の減少，血中炎症性マーカーの上昇および血中アルブミン値の低下を示し，臨床におけるがん悪液質研究の診断基準を反映したことから，有用な悪液質研究モデルとなりうることが考えられた。

さらに体重あたりのカロリー消費が高く，筋肉分解因子の亢進も確認されたことから，われわれの作製したがん悪液質モデルラットは，亢進したエネルギー消費が悪液質症状をさらに増悪させることを示唆するモデルであると考えられた。また，本モデルでは摂食量が低下しているにもかかわらず，摂食を亢進させるグレリンが血中で高値を示した。本モデルへのグレリン投与にても摂食行動が回復しなかったのは，グレリン抵抗性が惹起されていることが考えられた。悪液質を有するがん患者はすでに血中グレリンが高値を示すことが多く，本モデルは類似した症状を表現するモデルであると考えられる。

六君子湯は，グレリン抵抗性を有する同モデルにおいて予防的投与，がん悪液質発症後からの治療的投与のいずれにおいても，摂食量低下を改善し体重低下を抑制した。そのメカニズムとしては，六君子湯の生薬成分である蒼朮によるGHS-Rシグナルの増強作用を介して行われるものと推定される。

## Ⅴ まとめ

ヒト胃がん腹膜播種性転移株85As2細胞を接種することにより，新しいがん悪液質動物モデルを作製した。同モデルは臨床の悪液質の診断基準[4]を反映し，がん悪液質の病態生理研究および治療薬の評価に応用可能であると考えられた。本悪液質モデルにおいては，エネルギー消費亢進やグレリン抵抗性が認められ，ヒトのがん悪液質の病態を反映することが考えられた。また六君子湯は，予防的および治療的いずれの投与においても悪液質改善効果を示したことから，臨床にお



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いてがん患者のQOL向上への貢献が期待できると考えられる。

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ORIGINAL ARTICLE

# Pain-releasing action of Platelet-activating factor (PAF) antagonists in neuropathic pain animal models and the mechanisms of action

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**Conflicts of interest**
None declared.

**Abbreviations**
BN 50739, tetrahydro-4,7,8,10methyl-(chloro-2phenyl)6[dimethoxy-3,4-phenylthio]methylthiocarbonyl-9pyrido [4′,3′-4,5]thieno[3,2-f] triazolo-1,2,4[4,3-a] diazepine-1,4; siRNA, small interfering RNA; TCV-309, 3-bromo-5-[N-phenyl-N-[2-[[2-1,2,3,4-tetrahydro-2- isoquinolyl-carbonyloxy)ethyl]carbamoyl]ethyl]carbamoyl]-1-propylpyridinium nitrate); WEB 2086, 3-[4-(2-chlorophenyl)-9-methyl-6H-thieno [3,2-f][1,2,4]triazolo-[4,3-a][1,4]-diazepin-2-yl]-1-(4-morpholinyl)-1-propanone.

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## Abstract

**Background:** Platelet-activating factor (PAF) has been implicated in the pathology of neuropathic pain. Previous studies reported that PAF receptor (PAF-R) antagonists have varied anti-allodynia effects by route of administration and nerve injury models in rats.
**Methods:** The present study elucidated the effectiveness of PAF antagonists against neuropathic pain in four different models of peripheral nerve injury and provided insights into the mode of anti-allodynia action.
**Results:** PAF antagonists, TCV-309, BN 50739 and WEB 2086 by intravenous (i.v.) and oral administration have potent and long-lasting anti-allodynia action in mice neuropathic pain models. Treatment with PAF antagonists before surgery delayed the initiation of allodynia until the effects of these treatments were abolished. Intrathecal (i.t.) injection of the PAF antagonists and siRNA against PAF receptor ameliorated allodynia. I.t. injection of the glycine receptor (GlyR)α3 siRNA reduced the anti-allodynia effect of PAF antagonists. This evidence suggests that the anti-allodynia effect of PAF antagonists is at least in part mediated by spinal relief of PAF-induced dysfunction of GlyRα3. An analysis of the mode of anti-allodynia action of TCV-309 *in vivo* revealed a competitive action against PAF shortly after the injection of TCV-309, converting to a non-competitive action later.
**Conclusions:** The present results revealed the effectiveness in anti-allodynia of PAF antagonists in different nerve injury models, and the unique mode of action; long-lasting anti-allodynia effects mediated by spinal GlyRα3 with a competitive manner at the initial stage and the following non-competitive manner of inhibition.

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**What's already known about this topic?**

- Previous studies reported that platelet-activating factor receptor (PAF-R) antagonists have varied anti-allodynia effects in rat nerve injury.

**What does this study add?**

- Profound anti-allodynia effects by systemic injection of PAF-R antagonists were found or by intrathecal injection of siRNA of PAF-R in four different nerve injury models in mice.
- The results included long-lasting effects with initially competitive and the following non-competitive manner of inhibition.

## 1. Introduction

Several lines of evidence support the role of platelet-activating factor (PAF) as an important mediator of inflammatory responses (Ishii and Shimizu, 2000; Doi et al., 2006). However, the relevance of PAF in the pathology of pain is less clear, although some studies showed that PAF injected into the rat hind paw caused hypersensitivity to noxious stimulation (Bonnet et al., 1981; Vargaftig and Ferreira, 1981; Dallob et al., 1987; Marotta et al., 2009), persistent pain behaviours resulting from tissue injury by locally injected formalin or capsaicin were reduced in mice lacking the PAF receptor (Tsuda et al., 2007).

We have previously suggested that PAF may be a mediator of neuropathic pain. PAF injection into the mice spinal cord caused thermal hyperalgesia and tactile allodynia; key symptoms of neuropathic pain arise from innocuous stimuli, which were blocked by PAF receptor antagonists (Morita et al., 2004, 2008a). Subsequent studies showed PAF receptor blockade reduced pain behaviours elicited in nerve injury models. A PAF receptor antagonist CV-3988 by injecting near the dorsal root ganglion (DRG) in rats or mice lacking PAF receptors showed a reduction in tactile allodynia following spinal nerve injury accompanied by suppression of up-regulation of tumour necrosis factor and interleukin-1β expression in the injured DRG (Hasegawa et al., 2010). DRG contains lyso-PAF-acetyltransferase/lysophosphatidylcholine acyltransferase 2 (LPCAT2) and PAF receptor mRNA was increased in the ipsilateral DRG after nerve injury (Hasegawa et al., 2010). LPCAT2 mRNA and PAF receptor mRNA were increased in spinal microglia after nerve injury in rat spared nerve injury (SNI) model and i.t. injection of PAF antagonist WEB 2086 until 9 days post surgery suppressed the development of mechanical allodynia in the model (Okubo et al., 2012). PAF has been implicated in the pathophysiology of delayed tissue damage after various forms of brain injury including ischaemia, hypoxia and trauma (Lindsberg et al., 1990; Faden and Halt, 1992; Hostettler and Carlson, 2002). The evidence suggests that PAF contributes to neural tissue damage and pain behaviour after nerve injury.

However, neuropathic pain develops diverse pathological profiles depending on the disease, and thus the effectiveness of treatment with drugs is not uniform among them. Delayed administration of WEB 2086 did not reverse the allodynia in rat SNI model (Okubo et al., 2012).

CV-3988 did not suppress allodynia by injecting it into the lumbar enlargement of the spinal cord, while it was suppressed by injection near L5 DRG in spinal nerve-injured rats (Hasegawa et al., 2010). Therefore, we examined the effectiveness of systemic administration of several PAF antagonists against neuropathic pain in several different models in mice including a partial sciatic nerve ligation injury model, a partial infraorbital nerve ligation model, a chronic constriction of the infraorbital nerve injury (CCI model) and streptozotocin (STZ)-induced diabetes model, and found that PAF antagonists, TCV-309, BN 50739 and WEB 2086 produced profound and long-lasting anti-allodynia effects in these models. We further investigated the mode of action of PAF antagonist-induced pain relief.

## 2. Materials and methods

### 2.1 Animals

Experiments were performed on adult male ddY mice (Kyudo, Kumamoto, Japan) that were 5 weeks old and weighed 25–30 g at the beginning of the study. Mice were housed at 22 ± 2 °C with free access to commercial food and tap water. All experimental procedures and animal handling were performed according to both the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society and the guidelines of Hiroshima University, Hiroshima, Japan. The animals were used for only one measurement in each experiment.

### 2.2 Animal models

#### 2.2.1 Preparation of partial sciatic nerve injury mice and painful diabetic neuropathy mice

The experiments were conducted using a previously reported methods (Morita et al., 2008b).

### 2.2.2 Infraorbital nerve injury mice

Mice were anaesthetized with pentobarbital (60 mg/kg, i.p.). The head of the mouse was fixed in a Narishige head holder and mounted in a Kopf stereotaxic frame. A midline scalp incision was made, exposing the skull and nasal bone. The edge of the orbit, formed by the maxillary, frontal, lacrimal and zygomatic bones, was dissected free. To give access to the infraorbital nerve, orbital contents were gently deflected with a cotton-tripped wooden rod. The infraorbital nerve was dissected free at its most rostral extent in the orbital cavity, just caudal to the infraorbital foramen. Partial ligation of the infraorbital nerve was performed by tying the distal one-third to one-half of the infraorbital nerve (Partial ligation model). Chronic constriction of the infraorbital nerve was performed by loosely tying around the infraorbital nerve (2 mm apart) using two chromic gut (6-0) ligatures. To obtain the desired degree of constriction, a criterion formulated by Bennett and Xie (1988) was applied: the ligations reduced the diameter of the nerve by a just noticeable amount and retarded it, but did not occlude circulation through the superficial vasculature (CCI model). The scalp incision was closed in layers using nylon sutures. In mice in the control group, the infraorbital nerve was exposed on one side using the same procedure. The exposed infraorbital nerve was not ligated. Tactile allodynia was evaluated by measuring the face withdrawal threshold in response to probing with a series of calibrated fine filaments. Two weeks following nerve ligation, von Frey withdrawal thresholds were measured and the mice received a single dose a PAF antagonist or a vehicle.

## 2.3 Measurement of touch-evoked tactile allodynia

Tactile allodynia was assessed by lightly stroking the injured leg with a paintbrush or evaluated by measuring the paw withdrawal threshold in response to probing with a series of calibrated fine filaments as reported previously (Morita et al., 2008a,b).

## 2.4 Knockdown of PAF receptor and GlyRα3 in the spinal cord

Knockdown of PAF receptor and GlyRα3 were performed according to previous report (Morita et al., 2008a). The hemagglutinating virus of the Japan (HVJ) envelope vector system (HVJ Envelope Vector Kit GenomONE; Ishihara Sangyo Kaisha, Ltd, Osaka, Japan) was used for *in vivo* siRNA transfer. This HVJ-Envelope Vector has been proven to be an effective oligodeoxynucleotide delivery system both *in vitro* and *in vivo* (Kaneda et al., 2002). siRNAs were incorporated into the HVJ-Envelope Vector according to the manufacturer's instructions. Briefly, after mixing 40 μl (1 assay unit, AU) of HVJ-Envelope Vector with 4 μl of the enclosing factor, the mixture was centrifuged (10,000 × *g*, 10 min, 4 °C), and the pellet suspended in 10 μl of buffer solution. Then, 10 μl of a mixture of three siRNAs solution (#1, #2 and #3, 1 μg/μl each) was added, and the mixture was kept on ice for 5 min. Sterile artificial cerebrospinal fluid (ACSF, 10 μl) containing synthetic siRNA duplexes (0.45 pmole/animal) was injected into the subarachnoid space between the L5 and L6 vertebrate of conscious mice. The sequences of the siRNA oligonucleotide (sense) were as follows: PAF receptor (#1, 5′-CACCUCAGUGAGAAGUUUUACAGCA-AG-3′; #2, 5′-ACCCUUCCAAGAAACUAAAUGAGAU-AG-3′; #3, 5′-CAACUUCCAUCAGGCUAUUAAUGAU-AG-3′; targeting sequences around position 1005 to 1029, 232 to 256, 893 to 917, respectively, in *pafr*, GenBank accession no. D5087); GlyRα3 (#1, 5′-AGGUUUCGGCGAAAGAGAAAGAAUA-AG-3′; #2, 5′-GGUACUGCACUAAACACUACAAUAC-AG-3′; #3, 5′-CCUUAGGCAUGAAGACAUUCAUCAU-AG-3′; targeting sequences around position 1124 to 1148, 765 to 789, 1438 to 1462, respectively, in *glyra3*, GenBank accession no. NM_080438). Moreover, mismatched siRNA with three or four nucleotide mismatches was prepared to examine nonspecific effects of siRNA duplexes (PAF receptor siRNA#4, 5′-ACUCUGCCAAGAGACUACAUGAGAU-AG-3′; GlyRα3 siRNA#4, 5′-GAUACUGCACUACACACUACGAUAC-AG-3′). These selected sequences were also submitted to a BLAST search (Bioinformatics Center Institute for Chemical Research, Kyoto University, Japan) against the mouse genome sequence to ensure that only one gene in the mouse genome was targeted. siRNAs were purchased from iGENE Therapeutics Inc. (Tsukuba, Japan).

## 2.5 Experimental procedures

The first experiment examined whether various PAF antagonist had a profound anti-allodynia action in the present experimental neuropathic pain model. Following habituation and baseline testing, all mice received nerve injury as described above and the tactile allodynia was evaluated using paintbrush and von Frey tests. On a given test day, tactile allodynia was performed in all mice, and those displaying similar levels of tactile allodynia were randomized then divided into several groups. Mice then received intravenous (i.v.), i.t. or oral (per os, p.o.) injection of TCV-309, BN 50739, WEB 2086 or an equivalent volume of vehicle for drugs. Tactile allodynia was assessed before and after the i.t., i.v., p.o, injection.

A separate group of mice was used in the second experiment to examine the mode of anti-allodynia action of TCV-309 *in vivo*. Following habituation and four baseline measurements, mice were divided into several groups based on their responses to paintbrush and von Frey stimulation, providing those groups with similar average baseline mechanical sensitivity. On a given test day, TCV-309 (0.1 mg/kg) was intravenously injected 30 min after i.t. injection of 10 pg, 0.1 ng or 1 ng/mouse of PAF. The effect of TCV-309 pretreatment on allodynia induced by PAF was also examined. TCV-309 (0.1 mg/kg) was intravenously injected 30 min or 3 days before i.t. injection of various doses (0.001–

1000 pg/mouse) of PAF. Tactile allodynia was assessed before and after the i.t., i.v. injection.

Another group of mice received spinal transfer of siRNA against the PAF receptor or GlyRα3 to examine the involvement of the spinal PAF receptor and GlyRα3 in the PAF antagonist-induced anti-allodynia action in neuropathic pain models. Following habituation and baseline testing, all mice received nerve ligation as described above and the emergence of mechanical hypersensitivity was measured. On day 12 post surgery, tactile allodynia was performed in all mice, and those displaying similar levels of tactile allodynia were randomized then divided into several groups. Mice then received i.t. injection of either siRNAs, mutant siRNA, or HVJ-E vector alone. The effect of knockdown of the spinal PAF receptor on established nerve injury assessed tactile allodynia was examined. At 3 days after the knockdown of GlyRα3 by siRNA, the anti-allodynia response to TCV-309 was examined. The effect of a prophylactic PAF antagonist and knockdown of the spinal PAF receptor on the development of nerve injury tactile allodynia was also examined. In the prophylactic paradigm, following habituation and baseline testing, mice were divided into several groups based on their responses to both paintbrush and von Frey stimulation, providing those groups with similar baseline mechanical sensitivity. Mice then received i.v. injection of TCV-309 (100 μg/kg), BN 50739 (100 μg/kg), WEB 2086 (100 μg/kg) or a vehicle, or i.t. injection of siRNA or a mutant siRNA of PAF receptor mRNA or an HVJ-E vector alone 30 min or 3 days before surgery (at time 0). Tactile allodynia was assessed before and after the nerve ligation. Knockdown of the PAF receptor or GlyRα3 protein after spinal transfection of the PAF receptor or GlyRα3 siRNA were confirmed by immunoblotting analysis.

Throughout the experiment, behavioral testing was performed under blind conditions by a single experimenter. Injections were performed blind by another person.

### 2.6 Materials

PAF (1-*O*-hexadecyl-2-acetl-*sn*-glycero-3-phosphocholne), gabapentin (1-(aminomethyl)-cyclohexaneacetic acid) and STZ were obtained from Sigma-Aldrich (St. Louis, MO, USA). WEB 2086 (3-[4-(2-chlorophenyl)-9-methyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo- [4,3-*a*][1,4]-diazepin-2-yl]-1-(4-morpholinyl)-1-propanone) was obtained from Tocris Bioscience (Ellisville, MO, USA). TCV-309 (3-bromo-5-[*N*-phenyl-*N*-[2-[[2-(1,2,3,4-tetrahydro-2-isoquinolyl- carbonyloxy)ethyl]carbamoyl]ethyl]carbamoyl]-1-propylpyridinium nitrate), and BN 50739 (tetrahydro-4,7,8,10methyl-(chloro-2phenyl)6[dimethoxy-3,4-phenylthio]methylthio-carbonyl-9 pyrido[4′,3′-4,5]thieno[3,2-*f*]triazolo-1,2,4[4,3-*a*]diazepine-1,4) were donated from Takeda Pharmaceutical Co., and Institute Henri Beaufour, respectively.

BN 50739 was dissolved in a solvent containing 25% 2-hydroxypropyl-β-cyclodextrin (Sigma/RBI, Natick, MA, USA) and distilled water, pH adjusted to ~6 using 1 N NaOH, and diluted appropriately with ACSF or saline. PAF was dissolved in ethanol, which was then removed from an aliquot of this solution in a siliconized tube by introducing nitrogen gas into the tube. The PAF was then dissolved in 0.05% fatty acid-free bovine serum albumin containing ACSF. Other reagents were dissolved in ACSF or saline. ACSF composition (in mM) was NaCl 142, KCl 5, $CaCl_2 \cdot 2H_2O$ 2, $MgCl_2 \cdot 6H_2O$ 2, $NaH_2PO_4$ 1.25, D-glucose 10, HEPES 10, pH 7.4.

### 2.7 Data analysis

Data are expressed as the mean ± standard error of the mean (SEM) for each treatment group. Values are presented as an average allodynia score or withdrawal threshold at each time point during the time-course study. In other studies, allodynia was assessed every 5 min over a 60-min period (12 trials) and the values were expressed as the average % maximum possible cumulative score (possible cumulative maximum score: 2/mouse × 12 trials = 24), as described previously (Morita et al., 2004).

Regarding the statistical analyses of tactile allodynia, comparisons of allodynia scores and the withdrawal threshold of the differences between the drug-treated groups and the vehicle-treated group were evaluated using Dunnett's Multiple Comparison Test or unpaired *t*-test using GraphPad Prism version 4.0 (GraphPad Software Inc., San Diego, CA, USA).

## 3. Results

### 3.1 Effects of systemic administration of TCV-309, BN 50739 and WEB 2086, specific PAF receptor antagonists, on tactile allodynia in various neuropathic pain models of mice

Effects of PAF antagonists on allodynia induced by partial sciatic nerve ligation injury, partial infraorbital nerve ligation, CCI model and STZ-induced diabetes in mice were examined. TCV-309 with a range of 10~100 μg/kg by i.v and 30 μg~1 mg/kg by p.o. dose-dependently increased the withdrawal threshold at 10 to 15 days after partial ligation of the sciatic nerve in mice (Fig. 1A and B). The effects of TCV-309 were quite long lasting, for instance, TCV-309 ameliorated mechanical allodynia by a single injection of 10, 30 or 100 μg/kg, i.v. with a peak effect at 1 to 2 days after the injection until 3, 5 and 7 days, respectively. TCV-309 up to 1 mg/kg did not affect general behaviour or motor function estimated by RotaRod test (data not shown). BN 50739 and WEB 2086 also produced a long-lasting anti-allodynia effect with a similar range of doses (Table 1). The effect of these antagonists were much more potent and long lasting in comparison with the anti-allodynia effect of gabapentin 75 mg/kg, i.v. for which the anti-allodynia effect disappeared

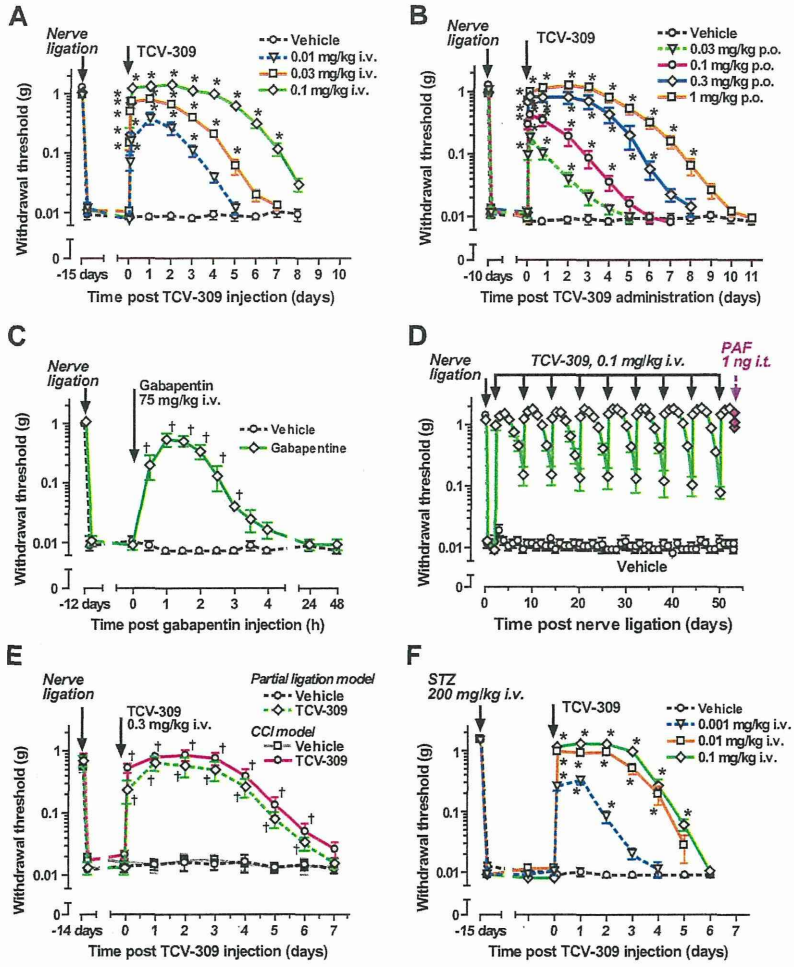


**Figure 1** (A, B) Systemic administration of TCV-309 ameliorated tactile allodynia assessed after partial sciatic nerve ligation injury in the mice. Tactile allodynia was assessed by measuring the paw withdrawal threshold in response to probing with von Frey hairs. TCV-309 was administered intravenously (A) or orally (p.o., B) at time 0. Experiments were performed at 10–15 days after the nerve ligation. Data are expressed as the mean ± standard error of the mean (SEM) $n = 7$–15 mice per group. Control mice received injections with a vehicle: saline. Tactile allodynia that developed after partial sciatic nerve ligation injury in mice was not affected by vehicle treatments. $*p < 0.05$ compared with the corresponding control (vehicle injection) values, as determined by analysis of variance followed by Dunnett's Multiple Comparison Test. (C) Intravenous injection of gabapentin ameliorated tactile allodynia assessed after partial sciatic nerve ligation injury in the mice. Gabapentin (75 mg/kg) was administered intravenously at time 0. Experiments were performed at 12 days after the nerve ligation. Data are expressed as the mean ± SEM $n = 7$ mice per group. Control mice received injections with a vehicle: saline. Tactile allodynia that developed after partial sciatic nerve ligation injury in mice was not affected by vehicle treatments. $\dagger p < 0.01$ compared with the corresponding control (vehicle injection) values, as determined by analysis of variance followed by an unpaired *t*-test. (D) Reproduction of the anti-allodynia effect of TCV-309 by repeated injection of TCV-309 in partial sciatic nerve ligation injury mice. TCV-309 was injected intravenously at 2, 8, 14, 20, 26, 32, 38, 44, 50 days after surgery. Tactile allodynia was assessed in mice using von Frey hairs (◊) on the ipsilateral paws 3 h and 1, 2 and 3 days after TCV-309 injection. Platelet-activating factor was administered intrathecally 3 days after the last injection of TCV-309. Preoperative basal values were obtained on day 0. Data are expressed as the mean ± SEM $n = 10$ mice per group. Control mice received injections with a vehicle: saline. (E) Intravenous injection of TCV-309 ameliorated tactile allodynia that developed in partial infraorbital nerve ligation model and chronic constriction of the infraorbital nerve injury (CCI) model mice. The experiments were carried out 14–20 days after the infraorbital nerve injury. Tactile allodynia was evaluated by measuring the face withdrawal threshold in response to probing with a series of calibrated fine filaments. TCV-309 (0.3 mg/kg) was administered intravenously at time 0. Data are expressed as the mean ± SEM $n = 6$–10 mice per group. Control mice received injections with a vehicle: saline. Tactile allodynia that developed after infraorbital nerve ligation injury in mice was not affected by vehicle treatment. $\dagger p < 0.05$ compared with the corresponding control (vehicle injection) values, as determined by analysis of variance followed by an unpaired *t*-test. (F) Intravenous injection of TCV-309 ameliorated tactile allodynia developed in streptozotocin (STZ)-induced painful diabetic neuropathy mice. The experiments were carried out 10–40 days after the injection of STZ or a vehicle. Tactile allodynia was assessed by measuring the paw withdrawal threshold in response to probing with von Frey hairs. TCV-309 was administered intravenously at time 0. Data are expressed as the mean ± SEM $n = 13$ mice per group. Control mice received injections with a vehicle: saline. Tactile allodynia that developed after STZ injection in mice was not affected by vehicle treatment. $*p < 0.05$ compared with the corresponding control (vehicle injection) values, as determined by analysis of variance followed by Dunnett's Multiple Comparison Test.

**Table 1** Effects of platelet-activating factor antagonists on tactile allodynia in various neuropathic pain models in mice.

| | Paintbrush test | | | | Von Frey test | | | |
|---|---|---|---|---|---|---|---|---|
| | $ED_{50}$ (μg/kg) | | Duration (days) | | $ED_{50}$ (μg/kg) | | Duration (days) | |
| | | | 0.1 μg/kg | 0.3 μg/kg | | | 0.1 μg/kg | 0.3 μg/kg |
| | i.v. | p.o. | i.v. | p.o. | i.v. | p.o. | i.v. | p.o. |
| Nerve ligation model | | | | | | | | |
| TCV-309 | 7.2 | 50.0 | 7 | 7 | 10.8 | 110.0 | 8 | 7 |
| BN 50739 | 13.0 | 29.6 | 7 | 7 | 14.5 | 33.4 | 8 | 7 |
| WEB 2086 | 4.5 | 30.6 | 6 | 8 | 8.4 | 67.1 | 6 | 8 |
| STZ diabetic model | | | | | | | | |
| TCV-309 | 1.5 | 16.0 | 5 | 7 | 5.8 | 38.0 | 5 | 7 |
| BN 50739 | 30.0 | 77.0 | 5 | 6 | 110.0 | 125.0 | 6 | 6 |
| WEB 2086 | 17.0 | 100.0 | 5 | 5 | 67.0 | 200.0 | 6 | 5 |

Nerve ligation model: partial sciatic nerve ligation injury model. STZ diabetic model: streptozotocin (STZ, 200 mg/kg i.v.)-induced painful diabetic neuropathy model. $ED_{50}$ values were estimated from the peak response by using least-squares linear regression. Duration: values are time during significant reduction of allodynia score and rise of withdrawal threshold. Data are expressed as the mean ($n$ = 7–15 mice per group).

within 4 h after i.v. injection (Fig. 1C). As the anti-allodynia effect of TCV-309 0.1 mg/kg, i.v. lasted for 7 days, the effect of the repeated administration every 6 days was examined. The potency of TCV-309 was similar in nine trials over 50 days without any refractory effect (Fig. 1D). I.t. injection of PAF at 1 ng produced a marked allodynia response (data not shown). When PAF was injected 3 days after the injection of the last TCV-309, it failed to produce allodynia, suggesting the effectiveness of TCV-309 as a PAF antagonist. The results show the PAF antagonists have potent anti-allodynia effects at very low doses and can avoid repeat treatment. However, one difficulty of clinically treating neuropathic pain with drugs is the difference in the effectiveness of drugs on different causes of pain. Therefore, the effectiveness of PAF antagonists on mechanical allodynia in other models such as a partial infraorbital nerve ligation model, a CCI model and a STZ-induced diabetes model were examined. The withdrawal threshold constantly decreased 10 days after the operation in both the partial infraorbital nerve ligation model and CCI model. Fourteen days after the operation, TCV-309 at 0.1 mg/kg, i.v. effectively increased the withdrawal threshold in both partial infraorbital nerve ligation model and the CCI model shortly after the injection and the effect lasted over 5 days (Fig. 1E). The anti-allodynia action of TCV-309, BN 50739 and WEB 2086 by i.v. injection in the STZ-induced diabetic model is shown in Fig. 1F. Anti-allodynia effects were obtained with a similar order of drugs in the nerve ligation models. The $ED_{50}$ values of TCV-309, BN 50739 and WEB 2086 administered by the i.v. and p.o. routes are summarized in Table 1. In the diabetic neuropathic model, TCV-309 was the most effective of the three drugs as shown by the low $ED_{50}$ value assessed by either the allodynia score or withdrawal threshold. However, the rank order of these drugs varied with the model and route of administration.

### 3.2 Effects of blockade of spinal PAF receptors on allodynia induced by partial ligation of the sciatic nerve and STZ-induced diabetes in mice

To elucidate the site of anti-allodynia action of PAF receptor antagonists, effects of i.t. injection of TCV-309, BN 50739 and WEB 2086, and also interference in the expression of spinal PAF receptors using siRNA of PAF receptor mRNA were examined. Spinal administration of 10 pg of these drugs immediately ameliorated allodynia (Fig. 2A). The evidence suggests that the site of action of PAF antagonists to produce an anti-allodynia effect involves at least the level of spinal cord. Knockdown of the expression of spinal PAF receptor protein was achieved by intrathecal transfer of PAF receptor siRNA in mice. As previously reported, a significant reduction in PAF receptor expression in normal mice and mice 15 days after surgery on the sciatic nerve achieved 37.5 ± 5.4% and 29.9 ± 4.9% of the control at 3 days after siRNA transfection, respectively, while the expression was not altered by the HVJ-E vector alone or mutant siRNA (Morita et al., 2008a). I.t. injection of PAF (0.1 pg) failed to evoke allodynia in siRNA-treated mice, while it induced allodynia in mice treated with mutant siRNA and the HVJ-E vector, as well as in vehicle-treated mice (these data are not shown). Thus, the knockdown of PAF receptor function by siRNA was established. The withdrawal threshold increased with the peak effect at 2 to 3 days after siRNA transfection,

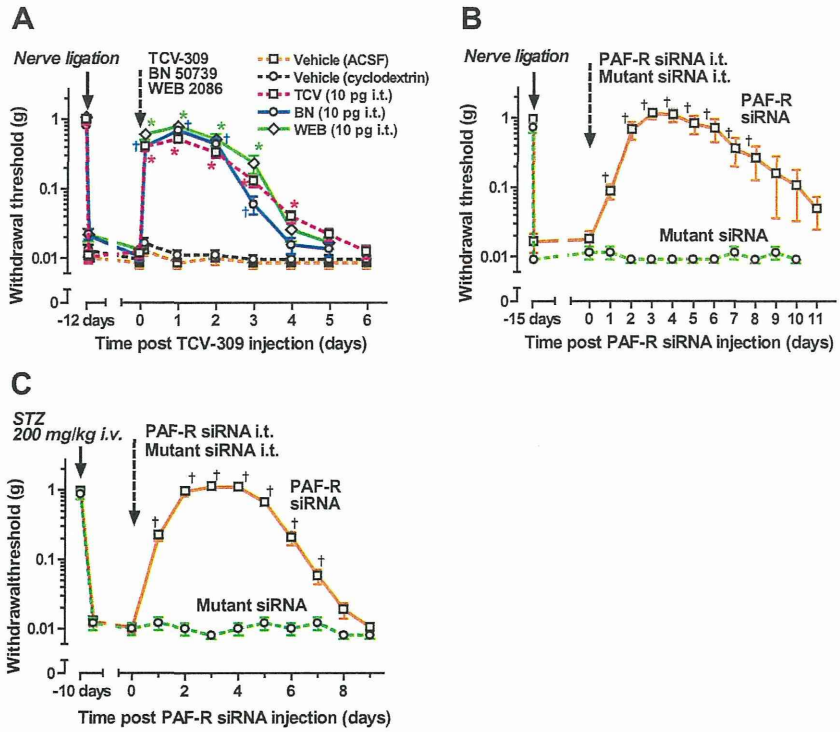


**Figure 2** (A) Intrathecal administered of platelet-activating factor (PAF) antagonists ameliorated tactile allodynia assessed after partial sciatic nerve ligation injury in the mice. Tactile allodynia was assessed by measuring the paw withdrawal threshold in response to probing with von Frey hairs. TCV-309 (10 pg/mouse), BN 50739 (10 pg/mouse), WEB 2086 (10 pg/mouse) or their vehicle were injected intrathecally at time 0. Experiments were performed at 12 days after the nerve ligation. Data are expressed as the mean ± standard error of the mean (SEM) $n = 6$–12 mice per group. Control mice received injections with a vehicle: ACSF or 25% 2-hydroxypropyl-β-cyclodextrin (cyclodextrin). Tactile allodynia that developed after partial sciatic nerve ligation injury in mice was not affected by vehicle treatment (not shown). *$p < 0.05$ compared with the corresponding control [vehicle (ACSF) injection] values, as determined by analysis of variance followed by Dunnett's Multiple Comparison Test. †$p < 0.01$ compared with the corresponding control (vehicle (cyclodextrin) injection) values, as determined by analysis of variance followed by an unpaired *t*-test. (B) Knockdown of spinal PAF receptor by siRNA ameliorated tactile allodynia assessed after partial sciatic nerve ligation injury in the mice. Tactile allodynia was assessed after partial sciatic nerve ligation injury in mice using von Frey hairs on the ipsilateral paws. siRNA or mutant siRNA of PAF receptor (PAF-R) mRNA were transfected into the spinal cord 15 days after nerve ligation. Data are expressed as the mean ± SEM ($n = 6$–8 mice per group). †$p < 0.05$ compared with the corresponding control (mutant siRNA injection) values, as determined by analysis of variance followed by an unpaired *t*-test. (C) Knockdown of spinal PAF receptor by siRNA ameliorated tactile allodynia that developed in streptozotocin (STZ)-induced diabetic mice. Tactile allodynia was evaluated by measuring the paw withdrawal threshold in response to probing with von Frey hairs. siRNA of PAF receptor (PAF-R) mRNA were transfected into the spinal cord 10 days after the treatment with STZ. Data are expressed as the mean ± SEM $n = 10$ mice per group. †$p < 0.01$ compared with the corresponding control (mutant siRNA injection) values, as determined by analysis of variance followed by an unpaired *t*-test.

while the anti-allodynia action gradually disappeared over 9 days in sciatic nerve-injured mice (Fig. 2B). Injection of mutant siRNA had no effect on the development of allodynia (Fig. 2B). Knockdown of the PAF receptor also reduced the allodynia response in STZ-induced neuropathic mice (Fig. 2C). The results further support the spinal site of anti-allodynia action of the PAF receptor blockade.

### 3.3 Effects of spinal GlyRα3 knockdown

We previously reported that intrathecally injected PAF-induced allodynia was mediated through glutamate-NO-cGMP and that this effect was blocked by knockdown of spinal GlyRα3. Therefore, the effects of knockdown of spinal GlyRα3 on the expression of tactile allodynia and PAF antagonist-induced anti-allodynia action were examined (Fig. 3). Fourteen days after sciatic nerve ligation, the allodynia score was maximally developed (maximal score is 2) and thus further increases in the score were not possible by transfection of GlyRα3 siRNA (Fig. 3A). TCV-309 at 100 μg/kg, i.v., more than the largest dose to produce an anti-allodynia effect in naïve animals (Fig. 1A) failed to ameliorate the allodynia in mice transfected with GlyRα3 siRNA, while it produced a profound

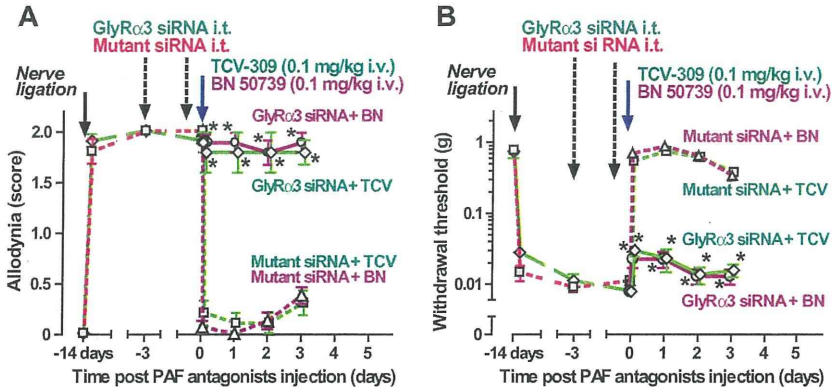


**Figure 3** Effect of knockdown of spinal GlyRα3 by siRNA on the anti-allodynia effect of PAF antagonists. Tactile allodynia was assessed after partial sciatic nerve ligation injury in mice using a paintbrush (A) or von Frey hairs (B) on the ipsilateral paws. siRNA (solid line) or mutant siRNA (dotted line) of GlyRα3 mRNA were transfected into the spinal cord 3 days and 3 h before the intravenous injection of TCV-309 (0.1 mg/kg) or BN 50739 (0.1 mg/kg). The experiments were carried out 11 days after nerve ligation. Data are expressed as the mean ± standard error of the mean $n = 10$ mice per group. $^*p < 0.01$ compared with the corresponding control (mutant siRNA injection) values, as determined by analysis of variance followed by an unpaired *t*-test.

anti-allodynia effect in mutant siRNA-treated mice (Fig. 3A). Transfection with GlyRα3 siRNA, but not mutant siRNA, slightly further reduced the withdrawal threshold and markedly reduced the anti-allodynia effect of TCV-309 (Fig. 3B). The anti-allodynia effect of BN 50739 was also reduced in GlyRα3 siRNA-transfected mice (Fig. 3B). Knockdown of GlyRα1 did not affect the effect of TCV-309 (data not shown). The evidence suggests that TCV-309 may exert its anti-allodynia action by antagonizing the reduction of GlyRα3-mediated inhibitory control of pain signal transduction by PAF in the spinal cord.

Glycine transporter (GlyT) inhibitors have a potent anti-allodynia effect in neuropathic pain models and spinal GlyRα3 has been implicated in the anti-allodynia action (Morita et al., 2008b; Dohi et al., 2009). However, the anti-allodynia action of GlyT inhibitors was stage-dependent on development of neuropathy, for instance, they did not block the developing allodynia response over 3 to 4 days after nerve injury and then produced a marked anti-alllodynia effect in established neuropathy in a partial sciatic nerve ligation model (Morita et al., 2008b). Although the present study showed the anti-allodynia effects by blocking PAF receptors in the established stage of neuropathy, whether or not blocking PAF receptors prior to surgery is effective was also examined. In control mice, almost a maximum allodynia score and a profound decrease in withdrawal threshold were observed shortly after nerve ligation. When PAF antagonists were injected intravenously 30 min before surgery, allodynia responses were not initiated until 4 days post surgery and allodynia gradually appeared and reached a stable state 8 days post surgery (Fig. 4A). The periods with suppressed appearance of allodynia by PAF antagonists post surgery almost corresponded with the effective periods of these antagonists. When treatment with PAF receptor siRNA was given 3 days before surgery, the initiation of allodynia started from 5 days post surgery, while it started shortly after surgery in mutant siRNA-treated mice (Fig. 4B). These results showed that PAF antagonists exert an anti-allodynia action against initiation of allodynia, as well as in an established state of allodynia.

### 3.4 Mode of anti-allodynia action of PAF antagonists

There is a striking difference in the duration of anti-allodynia action between PAF antagonists and gabapentin. To explore the long-acting effect of PAF antagonists, the dose-dependent mode of action of PAF antagonists against PAF-induced allodynia *in vivo* was analysed using TCV-309.

When TCV-309 (100 μg/kg, i.v.) was given at 30 min after 10 pg of PAF, i.t., the allodynia score decreased for 60 min after TCV-309 injection. The anti-allodynia potency of TCV-309 decreased by increasing concentration of PAF 10 pg to 1 ng of PAF (Fig. 5A). However, the anti-allodynia potency of TCV-309 gradually intensified as a function of time after TCV-309 treatment, for instance, the anti-allodynia potency of TCV-309 against PAF 1 ng was slight for 60 min, but gradually intensified and reached the maximum at 12 h after TCV-309 injection (Fig. 5A). These results suggest that TCV-309 has a different mechanism of action depending on the time after the injection. This concept is further supported