Title: Involvement of substance P in the peripheral neuropathy induced by paclitaxel but not by oxaliplatin

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Running Title: Involvement of substance P in paclitaxel-induced neuropathy

List of abbreviations:

ANOVA, analysis of variance; BNP7787, disodium 2,2'-dithio-bis-ethanesulfonate; CGRP, calcitonin gene-related peptide; DRG, dorsal root ganglion; GR159897, 5-fluoro-3-[2-[4-methoxy-4-[(R)-phenylsulphinyl]methyl]-1-piperidinyl]ethyl]-1H-indole; L-732,138, N-acetyl-L-tryptophan 3,5-bis(trifluoromethyl)benzylester; Pt(dach)Cl2, dichloro(1,2-diaminocyclohexane)platinum; TRP, transient receptor potential

Number of text pages: 33;
Number of figures: 8;
Number of tables: 0;
Abstract: 242;
Introduction: 637;
Discussion: 1122
Number of references: 40

Section: Neuropharmacology

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Abstract

The painful peripheral neuropathy occurring frequently during the chemotherapy with paclitaxel or oxaliplatin is one of their dose-limiting factors. We previously reported that substance P is involved in the pathogenesis of pulmonary hypersensitivity reaction to paclitaxel in rats, and an antiallergic agent pemirolast reverses this reaction via the blockade of release of substance P. In the present study, we investigated the involvement of substance P in the paclitaxel-induced peripheral neuropathy, compared with that by oxaliplatin. In the von Frey and acetone tests in rats, repeated administration of paclitaxel (6 mg/kg, i.p., once a week for 4 weeks) or oxaliplatin (4 mg/kg, i.p., twice a week for 4 weeks) induced both mechanical allodynia and cold hyperalgesia. The paclitaxel-induced peripheral neuropathy was mostly reversed by the acute administration of pemirolast (0.1 and 1 mg/kg, p.o.). Moreover, co-administration of neurokinin NK1 (L-732,138, 100 μg/body, i.t.) and NK2 (GR159897, 100 μg/body, i.t.) receptor antagonists strongly reversed the paclitaxel-induced neuropathy. On the other hand, the oxaliplatin-induced peripheral neuropathy was not reversed by pemirolast. In the in vitro study using cultured adult rat dorsal root ganglion (DRG) neurons, paclitaxel (1000 ng/mL) significantly increased the release of substance P, and pemirolast (100 and 1000 nM) significantly inhibited this increase of substance P release. Oxaliplatin, by contrast, did not increase the release of substance P. These results suggest that substance P is involved in the paclitaxel-induced neuropathy, and the mechanism of its action is clearly different from that of oxaliplatin.
Introduction

Paclitaxel, an anticancer agent with a tubulin-stabilizing action, has widely been used for several malignancies, including ovarian and breast cancer, non-small cell lung carcinoma, and stomach cancer. Oxaliplatin, a platinum-based chemotherapeutic agent, is very important in the chemotherapy for metastatic colorectal cancer. Although the mechanisms of these antitumor effects are different, their uses are often limited because of the incidence of severe adverse reactions including peripheral neuropathy.

The paclitaxel-induced peripheral neuropathy is characterized by frequently occurring sensory neuropathy, such as dysesthesia, numbness, pain and thermohyperesthesia in the feet and hands, and usually mild motor neuropathy (Lee and Swain, 2006). Oxaliplatin causes severe acute and chronic peripheral neuropathies (Gamelin et al., 2002). Acute neuropathy is peculiar to oxaliplatin and includes acral paresthesias enhanced by exposure to cold. On the other hand, the chronic neuropathy is characterized by loss of sensory and motor function after long-term treatment.

In clinical trials, amifostine, glutamine, acetyl L-carnitine, BNP7787 (disodium 2,2’-dithio-bis-ethanesulfonate) and vitamin E have been tested for paclitaxel-induced neuropathy (Argyriou et al., 2005; Bianchi et al., 2005; Lorusso et al., 2003; Stubblefield et al., 2005; Takeda et al., 2002). However, they are not commonly used in the clinical setting because of low effectiveness. Furthermore, calcium and magnesium infusions have been tried to reduce the oxaliplatin-induced neuropathy (Gamelin et al., 2008). Gabapentin, an antiepileptic agent, has been reported to attenuate the anticancer drugs-induced peripheral neuropathy in rodents (Ling et al., 2007). Recently, this drug is recommended as first-line treatment for the neuropathic pain (Dworkin et al., 2007). However, a phase III randomized double-blind trial failed to demonstrate any benefit to
using gabapentin to treat symptoms of chemotherapy-induced peripheral neuropathy (Rao et al., 2007). Therefore, new agents strongly reducing the symptoms of neuropathy are required.

The use of paclitaxel is also limited by several hypersensitivity reactions. We previously reported that paclitaxel markedly increases substance P in plasma and bronchoalveolar lavage fluid in rats, and in plasma in patients with ovarian cancer (Itoh et al., 2004a; Sendo et al., 2004). Moreover, we have reported that pemirolast, an antiallergic agent, attenuates the paclitaxel-induced pulmonary hypersensitivity reactions through inhibition of the release of sensory nerve peptides including substance P, neurokinin A and calcitonin gene-related peptide (CGRP) in the rat bronchoalveolar lavage fluid (Itoh et al., 2004b), and that pemirolast also prevents the paclitaxel-induced hypersensitivity reactions in patients with ovarian cancer (Yahata et al., 2006). Thus, sensory nerve peptides such as substance P play an important role in the paclitaxel-induced hypersensitivity reactions.

Substance P is well known to be a neurotransmitter related to the pain (Harrison and Geppetti, 2001) and is synthesized in the dorsal root ganglion (DRG) (Vedder and Otten, 1991). Substance P is localized in high threshold nociceptive C-fibers and is released by noxious stimulus (Otsuka and Yoshioka, 1993), and substance P binds neurokinin NK₁ and NK₂ receptors (Regoli et al., 1988). Substance P is involved in the regulation of nociceptive information at the first sensory synapse in the spinal cord (De Koninck and Henry, 1991). Moreover, substance P and neurokinin NK₁ receptors have been reported to be involved in neuropathic pain in traumatic rats (Cahill and Coderre, 2002; Goff et al., 1998; Gonzalez et al., 2000; Vachon et al., 2004). Neurokinin NK₁ and NK₂ receptors have also been reported to be involved in
neuropathic pain in diabetic rats (Coudoré-Civiale et al., 2000). Recently it was reported that paclitaxel evokes the release of substance P from cultured rat DRG cells (Miyano et al., 2009). However, the involvement of substance P in the chemotherapy-induced peripheral neuropathy remains unknown. Therefore, we investigated the involvement of substance P in the paclitaxel-induced peripheral neuropathy, compared with that by oxaliplatin. In this study, pemrolast, L-732,138 (N-acetyl-L-tryptophan 3,5-bis(trifluoromethyl)benzylester), a selective neurokinin NK₁ receptor antagonist, and GR159897 (5-fluoro-3-[2-[4-methoxy-4-[[((R)-phenylsulphinyl)methyl]-1-piperidinyl]ethyl]-1H-indole), a selective neurokinin NK₂ receptor antagonist, were used to investigate the involvement of substance P.
Methods

Animals

Male Sprague-Dawley rats weighing 200–250 g (Kyudo Co., Saga, Japan) were used in the present study. Animals were housed in groups of four to five per cage, with lights on from 8:00 to 20:00 h. Animals had free access to food and water in their home cages. The experimental procedures were approved by the Committee for the Care and Use of Laboratory Animals at the Faculty of Medicine, Kyushu University.

Drugs

Paclitaxel (Taxol®; 6 mg/mL in Cremophor EL/ethanol 1:1) and oxaliplatin (Elplat®) were obtained from Bristol-Myers Squibb (Tokyo, Japan) and Yakult Co., Ltd. (Tokyo, Japan), respectively. Pemirolast was a generous gift from Mitsubishi Tanabe Pharma Factory Ltd. (Osaka, Japan). L-732,138 and GR159897 were purchased from Biomol International (Butler, PA, USA) and Tocris (Ellisville, MO, USA), respectively. Pemirolast was dissolved in distilled water. Oxaliplatin was dissolved in 5% glucose solution. L-732,138 and GR159897 were dissolved in 100% dimethyl sulfoxide (DMSO). The doses of these drugs were chosen based on previous reports (Cahill and Coderre, 2002; Jamieson et al., 2005; Kawashiri et al., 2009; Ling et al., 2007).

Behavioral studies

The procedure was a modification of that described previously (Kawashiri et al., 2009). Behavioral testing was performed blind with respect to drug administration. von Frey test for mechanical allodynia. Rats were placed in a clear plastic box (20 × 17 × 13 cm) with a wire mesh floor and allowed to habituate for 30 min prior to
testing, von Frey filaments (The Touch Test Sensory Evaluator Set; Linton Instrumentation, Norfolk, UK) ranging 1-15 g bending force were applied to the mid-plantar skin of each hind paw six times, with each application held for 6 s. Fifty percent paw withdrawal threshold was determined by the up-down method (Chaplan et al., 1994; Dixon 1980).

Acetone test for cold hyperalgesia. The acetone test was performed according to the method described by Flatters and Bennett (2004). Rats were placed in a clear plastic box (20 × 17 × 13 cm) with a wire mesh floor and allowed to habituate for 30 min prior to testing. Fifty microlitre of acetone (Wako Pure Chemical Ltd., Osaka, Japan) was sprayed onto the plantar skin of each hind paw three times with a Micro Sprayer® (Penn Century Inc., Philadelphia, PA, USA), and the number of withdrawal response was counted for 40 s from the start of the acetone spray.

To investigate the effects of paclitaxel and oxaliplatin on the nociceptive behaviors, two independent experiments were carried out.

**Effect of paclitaxel on mechanical and cold nociceptive threshold**

We investigated the effect of paclitaxel on mechanical and cold nociceptive threshold in the von Frey and acetone tests. Paclitaxel (6 mg/kg) or vehicle (Cremophor EL/ethanol 1:1) was injected i.p. once a week for 4 weeks (on Days 1, 8, 15, and 22). The von Frey test was performed before the first administration of paclitaxel (on Day 0) and on Days 5, 12, 19 and 26. The acetone test was performed immediately after the von Frey test.

**Effect of oxaliplatin on mechanical and cold nociceptive threshold**
We investigated the effect of oxaliplatin on the mechanical and cold nociceptive threshold in the von Frey and acetone tests. Oxaliplatin (4 mg/kg) or vehicle (5% glucose solution) was administered i.p. twice a week for 4 weeks (on Days 1, 2, 8, 9, 15, 16, 22 and 23). The von Frey test was performed before the first administration of oxaliplatin (on Day 0) and on Days 3, 10, 17 and 24. The acetone test was performed immediately after the von Frey test.

**Effect of pemirolast on the paclitaxel- or oxaliplatin-induced mechanical allodynia and cold hyperalgesia**

In the case of paclitaxel, we confirmed the incidence of peripheral neuropathy on Day 28 and carried out the drug evaluation on the following day. The von Frey test was performed immediately before (0 min) and 30, 60, 90, 120 and 180 min after oral administration of pemirolast. The acetone test was performed immediately after the von Frey test. In the case of oxaliplatin, we confirmed the incidence of mechanical allodynia and cold hyperalgesia on Days 24 and 3, respectively. We carried out the drug evaluation on the next day. The von Frey and acetone tests were performed immediately before (0 min) and 30, 60, 90, 120 and 180 min after oral administration of pemirolast.

**Effects of L-732,138 and GR159897 on the paclitaxel-induced mechanical allodynia and cold hyperalgesia**

We confirmed the incidence of peripheral neuropathy on Day 28 and carried out the drug evaluation on the next day. L-732,138 and GR159897 were administered intrathecal (i.t.) injection by direct lumbar puncture in a volume of 50 μL. The animals were anesthetized lightly with ether for intrathecal injection. Therefore, the pain
behavior was measured from 60 min after intrathecal injection when anesthesia was completely disappeared. The von Frey test was performed immediately before (0 min) and 60, 90, 120 and 180 min after administration of L-732,138 or GR159897. The acetone test was performed immediately after the von Frey test.

Preparation of rat DRG cells for assay of substance P release

L 4-5 DRG cells were removed from male Sprague-Dawley rats (6 weeks old) anesthetized with sodium pentobarbital and cultured. Ganglia were incubated with 0.125% (w/v) collagenase type 1 (Worthington Biochemical Corp, NJ, USA) at 37 °C for 90 min followed by 0.25% (w/v) trypsin-EDTA (Gibco BRL, USA) for 30 min. The DRG cells were grown in Dulbecco’s modified Eagle’s medium (MP Biomedicals Inc., Solon, OH, USA) supplemented with 100 unit/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (GIBCO BRL, Grand island, NY, USA, respectively) and 10% fetal bovine serum (Cell Culture Technology, Hannover, Germany). The cells (2 DRGs/well) were maintained at 37 °C in a water-saturated atmosphere with 5% CO₂ for 10 days.

Measurement of substance P release from cultured rat DRG neurons

DRG neurons were pretreated with or without pemirolast (10-1000 nM) in 1mL Hanks’ balanced salt solution (HBSS; in g/L: 8.00 NaCl, 0.40 KCl, 0.14 CaCl₂, 0.10 MgSO₄ 7H₂O, 0.10 MgCl₂ 6H₂O, 0.06 Na₂HPO₄ 2H₂O, 0.06 KH₂PO₄, 1.00 glucose, 0.35 NaHCO₃, pH 7.4) with peptidase inhibitor aprotinin (Wako Pure Chemical Ltd., Osaka, Japan) for 3 h at 37 °C in a water-saturated atmosphere with 5% CO₂. Then the medium is changed, and DRG neurons were treated with paclitaxel (1-1000 ng/mL) or oxaliplatin (0.01-100 μg/mL) with or without pemirolast (10-1000 nM) for 10 min at
37 °C. The content of substance P in the culture medium was measured by EIA kit (Cayman Chemical Co., Ann Arbor, MI, USA).

Data analysis

Values were expressed as the means ± S.E.M. The values were analyzed by the Student’s t-test, or one-way or two-way (with repeated measures) analysis of variance (ANOVA) followed by the Tukey-Kramer post hoc test (StatView; Abacus Concepts, Berkely, CA, USA) to determine differences among the groups. A probability level of $p < 0.05$ was accepted as statistically significant.
Results

Effects of paclitaxel and oxaliplatin in the von Frey and acetone tests

Before the first drug administration, there were no significant differences in all groups in 50% paw withdrawal threshold in the von Frey test and in number of withdrawal responses in the acetone test. In the von Frey test in paclitaxel-treated rats, repeated measures ANOVA revealed a significant time effect \(F(4, 44)=4.561, p < 0.01\), a significant drug × time interaction \(F(4, 44)=4.199, p < 0.01\), but no significant drug effect. Paclitaxel (6 mg/kg, i.p.) significantly reduced the 50% paw withdrawal threshold on Days 19 and 26 compared with Day 0 in the von Frey test \(p < 0.01\) by Tukey-Kramer post hoc test, Fig. 1A). In the acetone test, repeated measures ANOVA revealed a significant time effect \(F(4, 44)=9.940, p < 0.0001\), a significant drug × time interaction \(F(4, 44)=3.172, p < 0.05\), but no significant drug effect. Paclitaxel at the same dose significantly increased the number of withdrawal responses in the acetone test on Days 19 and 26 compared with Day 0 \(p < 0.01\) by Tukey-Kramer post hoc test, Fig. 1B).

In oxaliplatin-treated rats, repeated measures ANOVA revealed a significant drug effect \(F(1, 11)=12.121, p < 0.01\), a significant time effect \(F(4, 44)=2.605, p < 0.05\) and a significant drug × time interaction \(F(4, 44)=5.009, p < 0.01\) in the von Frey test. Oxaliplatin (4 mg/kg, i.p.) significantly reduced the 50% paw withdrawal threshold on Day 24 compared with Day 0 in the von Frey test \(p < 0.05\) by Tukey-Kramer post hoc test, Fig. 2A). In the acetone test, repeated measures ANOVA revealed a significant drug effect \(F(1, 11)=30.967, p < 0.001\), a significant time effect \(F(4, 44)=8.521, p < 0.0001\) and a significant drug × time interaction \(F(4, 44)=4.946,
Oxaliplatin at the same dose significantly increased the number of withdrawal responses in the acetone test on Days 3, 10, 17 and 24 compared with Day 0 (p < 0.01 by Tukey-Kramer post hoc test, Fig. 2B).

Effect of pemirolast on paclitaxel- or oxaliplatin-induced peripheral neuropathy

Paclitaxel (6 mg/kg, i.p.) significantly reduced the 50% paw withdrawal threshold in the von Frey test and increased the number of withdrawal responses in the acetone test compared with vehicle on Day 28 (p < 0.01 by Student’s t-test, Figs. 3A and 3B). Before administration of pemirolast, each group had equivalent 50% withdrawal threshold in the von Frey test and number of withdrawal responses in the acetone test. In the von Frey test, repeated measures ANOVA revealed a significant drug effect [F(3, 20)=3.693, p < 0.05], a significant time effect [F(5, 100)=20.082, p < 0.01] and a significant drug × time interaction [F(15, 100)=4.776, p < 0.01]. Pemirolast (0.1 and 1 mg/kg, p.o.) almost completely reversed the reduction of 50% paw withdrawal threshold by paclitaxel at 30 and 60 min after administration in the von Frey test (p < 0.05 or 0.01 by Tukey-Kramer post hoc test, Fig. 3A). At 120 min after administration, this effect of pemirolast was disappeared. In the acetone test, repeated measures ANOVA revealed a significant time effect [F(5, 100)=2.933, p < 0.05], but no significant drug effect or drug × time interaction. Pemirolast (1 mg/kg, p.o.) significantly reversed the increase of number of withdrawal responses by paclitaxel at 30 min after administration in the acetone test (p < 0.05 by Tukey-Kramer post hoc test, Fig. 3B). This effect of pemirolast was disappeared at 120 min after administration.

Oxaliplatin (4 mg/kg, i.p.) significantly reduced the 50% paw withdrawal threshold in the von Frey test on Day 24 (p < 0.01 by Student’s t-test, Fig. 4A) and
increased the number of withdrawal responses in the acetone test compared with vehicle on Day 3 and 24 \((p < 0.01\) by Student’s \(t\)-test, Figs. 4B and 4C). Before administration of pemirolast, each group had equivalent 50% withdrawal threshold in the von Frey test and number of withdrawal responses in the acetone test. In the von Frey test, repeated measures ANOVA revealed a significant time effect \([F(5, 140)=3.990, p < 0.01]\), but no significant drug effect or drug \(\times\) time interaction. Pemirolast had no effect on the reduction of 50% paw withdrawal threshold by oxaliplatin in the von Frey test (Fig. 4A).

In the acetone test, repeated measures ANOVA revealed no significant time effect, drug effect or drug \(\times\) time interaction. Pemirolast did not affect the increase of number of withdrawal responses by oxaliplatin in the acetone test (Figs. 4B and 4C).

**Effects of neurokinin NK1 and NK2 receptor antagonists on paclitaxel-induced peripheral neuropathy**

In the von Frey test in rats treated with selective neurokinin NK1 receptor antagonist L-732,138, repeated measures ANOVA revealed a significant time effect \([F(4, 92)=9.675, p < 0.01]\), a significant drug \(\times\) time interaction \([F(12, 92)=2.261, p < 0.05]\), but no significant drug effect. L-732,138 (100 μg/body, i.t.) significantly reversed the reduction of 50% paw withdrawal threshold by paclitaxel at 60 min after administration in the von Frey test \((p < 0.05\) by Tukey-Kramer post hoc test, Fig. 5A). This effect of L-732,138 was disappeared at 120 min after administration. In the acetone test, repeated measures ANOVA revealed a significant time effect \([F(4, 92)=3.426, p < 0.05]\), but no significant drug effect or drug \(\times\) time interaction. L-732,138 at the same dose had no effect on the increase of number of withdrawal responses by paclitaxel in the acetone test (Fig. 5B).
In the von Frey test in rats treated with selective neurokinin NK₂ receptor antagonist GR159897, repeated measures ANOVA revealed a significant time effect \[F(4, 92)=3.543, p < 0.01\], a significant drug × time interaction \[F(12, 92)=2.520, p < 0.01\], but no significant drug effect. GR159897 (100 μg/body, i.t.) significantly reversed the reduction of 50% paw withdrawal threshold by paclitaxel at 60 min after administration in the von Frey test \((p < 0.05\) by Tukey-Kramer post hoc test, Fig. 6A). This effect of GR159897 was disappeared at 120 min after administration. In the acetone test, repeated measures ANOVA revealed a significant time effect \[F(4, 92)=4.090, p < 0.01\], but no significant drug effect or drug × time interaction. GR159897 at the same dose did not affect the increase of number of withdrawal responses by paclitaxel in the acetone test (Fig. 6B).

In the von Frey test in rats treated with co-administration of L-732,138 and GR159897, repeated measures ANOVA revealed a significant drug effect \[F(3, 23)=8.659, p < 0.01\], a significant time effect \[F(4, 92)=30.184, p < 0.01\] and a significant drug × time interaction \[F(12, 92)=5.704, p < 0.01\]. Co-administration of L-732,138 (100 μg/body, i.t.) and GR159897 (100 μg/body, i.t.) completely reversed the reduction of 50% paw withdrawal threshold by paclitaxel at 60 and 90 min after administration in the von Frey test, and this effect was continued until 180 min \((p < 0.05\) or 0.01 by Tukey-Kramer post hoc test, Fig. 7A). In the acetone test, repeated measures ANOVA revealed a significant time effect \[F(4, 92)=4.456, p < 0.01\], a significant drug × time interaction \[F(12, 92)=1.981, p < 0.05\], but no significant drug effect. Co-administration of L-732,138 and GR159897 at the same dose significantly reversed the increase of number of withdrawal responses by paclitaxel at 60 min after administration in the acetone test \((p < 0.01\) by Tukey-Kramer post hoc test, Fig. 7B).
This effect was disappeared at 120 min after administration.

**Effects of paclitaxel and oxaliplatin on release of substance P from cultured adult rat DRG neurons**

Paclitaxel (1000 ng/mL) significantly increased the release of substance P from cultured DRG neurons ($p < 0.01$ by Tukey-Kramer post hoc test, Fig. 8A). Moreover, pemirolast (100 and 1000 nM) significantly inhibited the increase of substance P release induced by paclitaxel ($p < 0.05$ by Tukey-Kramer post hoc test, Fig. 8B). On the other hand, oxaliplatin (0.01-100 µg/mL) did not increase the release of substance P (Fig. 8C).
Discussion

The present study showed that paclitaxel increases the release of substance P from cultured adult rat DRG neurons consistently with the recent report (Miyano et al., 2009). Apfel and colleagues reported that paclitaxel decreased the DRG cells content of substance P in mice (Apfel et al., 1991). Paclitaxel has been reported to evoke the release of substance P from cultured DRG cells by the extracellular Ca$^{2+}$ influx through transient receptor potential (TRP) channels (Miyano et al., 2009). Oxaliplatin, by contrast, did not increase the release of substance P. These findings suggest that the effect of oxaliplatin may be different from that of paclitaxel, and oxaliplatin may have no effect on pathways mediating substance P release.

Ling and colleagues reported an increase of substance P expression in the dorsal horn spinal cord 24 h after single administration of oxaliplatin (6 mg/kg, i.p.) (Ling et al., 2007). On the other hand, our data in this study revealed that oxaliplatin (4 mg/kg, i.p. twice a week for 4 weeks) did not increase the release of substance P from DRG neurons. This discrepancy might be due to the difference of experimental methods such as administration schedule and measurement site.

We also found that pemirolast significantly inhibits this paclitaxel-induced increase of substance P release. Pemirolast inhibits not only the release of chemical mediators such as histamine and leukotriene but also the release of sensory nerve peptides including substance P, neurokinin A and CGRP (Itoh et al., 2004b). Pemirolast also has inhibitory effects of inositol 1,4,5-trisphosphate production, Ca$^{2+}$ mobilization and phosphodiesterase (Fujimiya et al., 1994; Yanagihara et al., 1988). Paclitaxel has been reported to evoke the release of substance P from cultured DRG cells by the
extracellular Ca\(^{2+}\) influx through TRP channels (Miyano et al., 2009). Therefore, pemirolast might reduce the paclitaxel-induced increase of substance P release from cultured DRG neurons by inhibiting the extracellular Ca\(^{2+}\) influx.

In the present study, we observed the mechanical allodynia (von Frey test) and cold hyperalgesia (acetone test) after paclitaxel or oxaliplatin administration, in consistent with our previous reports (Kawashiri et al., 2009; Sakurai et al., 2009). Paclitaxel induced the mechanical alldonyia and cold hyperalgesia on Days 19 and 26. We previously indicated that paclitaxel causes axonal degeneration of sciatic nerve on Days 25 (Kawashiri et al., 2009). Clinical studies have shown that the axonal degeneration of nerves is caused by paclitaxel, as well as a reduction of myelinated fiber density and the loss of large fibers (Lee and Swain, 2006). These neurodegenerations may contribute to the paclitaxel-induced neuropathy symptoms such as mechanical allodynia and cold hyperalgesia.

Oxaliplatin also caused the mechanical allodynia on Days 17 and 24 (at the same phase) in the present study. Oxaliplatin causes the damage of the cell bodies, the alterations in nucleus and nucleolus (Cavaletti et al., 2001; McKeage et al., 2001) and selective atrophy of subpopulation of DRG neurons (Jamieson et al., 2005) in animal models. Moreover, oxaliplatin induces the cell death and the inhibition of neurite outgrowth (Luo et al., 1999; Ta et al., 2006) in neuronal cells. The neurotoxicity of oxaliplatin for the DRG neurons correlates with platinum-DNA bindings (Ta et al., 2006). In our previous study, dichloro(1,2-diaminocyclohexane)platinum (Pt(dach)Cl\(_2\)), a metabolite of oxaliplatin, induced the mechanical allodynia on Days 17 and 24, but did not induce the cold hyperalgesia/allodynia (Sakurai et al., 2009). These findings suggest that the oxaliplatin-induced mechanical allodynia may be due to the
neurotoxicity of platinum. On the other hand, oxaliplatin caused the cold hyperalgesia from Day 3 (early phase). This phenomenon resembled the clinical symptom of the oxaliplatin-induced acute neuropathy. We recently indicated that oxalate, another metabolite of oxaliplatin, is involved in the cold hyperalgesia but not mechanical allodynia in the oxaliplatin-induced neuropathy, and that oxalate is the main causative agent in this cold hyperalgesia through chelating of Ca\(^{2+}\) and Mg\(^{2+}\) (Sakurai et al., 2009). Thus, the mechanism of its appearance seems to be clearly different from that of mechanical allodynia.

Interestingly, in this study, pemirolast almost completely relieved the paclitaxel-induced mechanical allodynia and cold hyperalgesia, whereas pemirolast had no effect on the oxaliplatin-induced neuropathy. Paclitaxel but not oxaliplatin increased the release of substance P from cultured adult rat DRG neurons. Moreover, pemirolast inhibited this paclitaxel-induced increase of substance P release. Taken together with these findings, the present results suggest that substance P may be involved in the neuropathy induced by paclitaxel but not oxaliplatin, and that pemirolast may relieve the paclitaxel-induced neuropathy by inhibiting the release of substance P. Thus, the difference in role of substance P between paclitaxel and oxaliplatin might be due to these drug’s different effects on the substance P release. In addition, pemirolast completely did not reverse the paclitaxel-induced increase of substance P release although pemirolast almost completely relieved the paclitaxel-induced neuropathy, suggesting the possible involvement of other neuropeptides such as CGRP in the ameliorative effect of pemirolast.

In the present study, pemirolast was orally injected. Since pemirolast has poor blood-brain barrier permeability, the ameliorative effect of pemirolast is probably due to
the peripheral response. This idea is supported by our result that pemirolast inhibited the paclitaxel-induced increase of substance P release from DRG neurons. Moreover, intrathecal injection of neurokinin NK₁ and NK₂ receptor antagonists strongly reversed the paclitaxel-induced neuropathy, indicating that these antagonists inhibited the pain behavior by blocking these receptors in the spinal cord. These findings suggest that substance P released from periphery is involved in the paclitaxel-induced neuropathy.

Substance P binds neurokinin NK₁ and NK₂ receptors (Regoli et al., 1988). Vachon and colleagues suggested a correlation between an increase of substance P and the development of mechanical allodynia induced by sciatic nerve cuff implantation (Vachon et al., 2004). Goff and colleagues reported that the chronic constriction injury (CCI)-induced increase in NK₁ receptor immunoreactivity paralleled the time course of mechanical behavioral hypersensitivity (Goff et al., 1998). In animal model of diabetic neuropathy, NK₁ and NK₂ receptor antagonists showed antinociceptive effect (Coudoré-Civiale et al., 2000). Moreover, NK₁ receptor antagonists have been reported to relieve the peripheral nerve injury-induced mechanical allodynia and cold hyperalgesia (Cahill andCoderre, 2002; Gonzalez et al., 2000). Dionne and colleagues clinically demonstrated that a NK₁ receptor antagonist relieves acute postoperative pain (Dionne et al., 1998). In the present study, we observed that co-administration of NK₁ and NK₂ receptor antagonists completely relieves the paclitaxel-induced peripheral neuropathy. Our findings suggest that NK₁ and NK₂ receptors play an important role in the paclitaxel-induced peripheral neuropathy, and these findings strongly confirm the involvement of substance P in the paclitaxel-induced neuropathy.

In conclusion, the present study revealed that substance P is involved in the paclitaxel-induced neuropathy, and the mechanism of its action is clearly different from
that of oxaliplatin. Finally, pemirolast is expected to be useful as a therapeutic drug for clinical paclitaxel-induced neuropathy.
Acknowledgements

The authors are grateful to Mitsubishi Tanabe Pharma Factory Ltd. (Osaka, Japan) for generously supplying the pemirolast.
Authorship Contributions

Participated in research design: Tatsushima, Egashira, Kawashiri, Mihara. Yano, Mishima, and Oishi

Conducted experiments: Tatsushima, Kawashiri, and Mihara.

Performed data analysis: Tatsushima, Egashira, and Kawashiri.

Wrote or contributed to the writing of the manuscript: Tatsushima, Egashira, Kawashiri, and Oishi.
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Footnotes

Part of this study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Nos. 21590285 and 22590242). We certify that there were no conflicts of interest in this work.
Legends for Figures

Figure 1. Mechanical allodynia in the von Frey test (A) and cold hyperalgesia in the acetone test (B) induced by paclitaxel in rats. Rats were treated with paclitaxel (6 mg/kg, i.p.) once a week for 4 weeks. Number of animals was shown in each parenthesis. Values are expressed as the mean ± S.E.M on Days 0, 5, 12, 19 and 26. **p < 0.01 compared with Day 0.

Figure 2. Mechanical allodynia in the von Frey test (A) and cold hyperalgesia in the acetone test (B) induced by oxaliplatin in rats. Rats were treated with oxaliplatin (4 mg/kg, i.p.) twice a week for 4 weeks. Number of animals was shown in each parenthesis. Values are expressed as the mean ± S.E.M on Days 0, 3, 10, 17 and 24. *p < 0.05, **p < 0.01 compared with Day 0.

Figure 3. Effect of pemirolast on mechanical allodynia in the von Frey test (A) and cold hyperalgesia in the acetone test (B) in paclitaxel-treated rats. Rats were treated with paclitaxel (6 mg/kg, i.p.) once a week for 4 weeks. Pemirolast (0.01-1 mg/kg) was administered orally. Number of animals was shown in each parenthesis. Values are expressed as the mean ± S.E.M. ††p < 0.01 compared with vehicle, *p < 0.05, **p < 0.01 compared with paclitaxel alone.

Figure 4. Effect of pemirolast on mechanical allodynia in the von Frey test (A) and cold hyperalgesia in the acetone test on 1st (B) and 4th weeks (C) in oxaliplatin-treated rats. Rats were treated with oxaliplatin (4 mg/kg, i.p.) twice a week for 4 weeks. Pemirolast
(0.01-1 mg/kg) was administered orally. Number of animals was shown in each parenthesis. Values are expressed as the mean ± S.E.M. ††p < 0.01 compared with vehicle.

Figure 5. Effect of L-732,138 on mechanical allodynia in the von Frey test (A) and cold hyperalgesia in the acetone test (B) in paclitaxel-treated rats. Rats were treated with paclitaxel (6 mg/kg, i.p.) once a week for 4 weeks. L-732,138 (10-100 μg/body) was administered i.t. Number of animals was shown in each parenthesis. Values are expressed as the mean ± S.E.M. ††p < 0.01 compared with vehicle, *p < 0.05 compared with paclitaxel alone.

Figure 6. Effect of GR159897 on mechanical allodynia in the von Frey test (A) and cold hyperalgesia in the acetone test (B) in paclitaxel-treated rats. Rats were treated with paclitaxel (6 mg/kg, i.p.) once a week for 4 weeks. GR159897 (10-100 μg/body) was administered i.t. Number of animals was shown in each parenthesis. Values are expressed as the mean ± S.E.M. ††p < 0.01 compared with vehicle, *p < 0.05 compared with paclitaxel alone.

Figure 7. Effect of co-administration of L732,138 and GR159897 on mechanical allodynia in the von Frey test (A) and cold hyperalgesia in the acetone test (B) in paclitaxel-treated rats. Rats were treated with paclitaxel (6 mg/kg, i.p.) once a week for 4 weeks. L-732,138 (100 μg/body) and GR159897 (100 μg/body) were administered i.t. Number of animals was shown in each parenthesis. Values are expressed as the mean ± S.E.M. ††p < 0.01 compared with vehicle, *p < 0.05, **p < 0.01 compared with
paclitaxel alone.

Figure 8. Release of substance P from cultured adult rat DRG neurons. DRG neurons were pretreated with or without pemirolast [10-1000 nM (B)] for 3 h in HBSS, followed by the treatment with paclitaxel [1-1000 ng/mL (A)] or oxaliplatin [0.01-100 μg/mL (C)] for 10 min at 37 °C. Values are expressed as the mean ± S.E.M of five experiments. **p < 0.01 compared with control, †p < 0.05 compared with paclitaxel alone.
Fig. 1.

(A) von Frey test

(B) Acetone test
Fig. 2.

(A) von Frey test

(B) Acetone test
Fig. 3.

(A) von Frey test

(B) Acetone test
Fig. 4.

(A) von Frey test

(B) Acetone test (1 week)

(C) Acetone test (4 week)
Fig. 5.
Fig. 6.
Fig. 7.
Fig. 8.