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Neurotropin reverses paclitaxel-induced neuropathy without affecting anti-tumour efficacy

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ABSTRACT

Paclitaxel is a commonly used anticancer drug, but it frequently causes peripheral neuropathy. Neurotropin, a non-protein extract from inflamed rabbit skin inoculated with vaccinia virus, has been used to treat various chronic painful conditions. In the present study, we investigated the effect of neurotropin on the paclitaxel-induced neuropathy in rats. Repeated administration of paclitaxel induced mechanical allodynia, cold hyperalgesia, and motor dysfunction. These neuropathies were mostly reversed by the repeated administration of neurotropin. Furthermore, neurotropin ameliorated the paclitaxel-induced axonal degeneration in cultured PC12 and rat dorsal root ganglion cells, and in rat sciatic nerve. In addition, neurotropin did not affect the microtubule aggregation or anti-tumour effect induced by paclitaxel in the tumour cell lines or tumour cells-implanted mice. These results suggest that neurotropin reverses the paclitaxel-induced neuropathy without affecting anti-tumour activity of paclitaxel, and therefore may be useful for the paclitaxel-induced neuropathy in clinical settings.

1. Introduction

Paclitaxel (Taxol®), an anticancer agent with a tubulin-stabilising action, is widely used for several malignancies, including ovarian and breast cancer, non-small cell lung carcinoma, and stomach cancer. However, its use is often limited because of the incidence of severe adverse reactions including a peripheral neuropathy.

The paclitaxel-induced peripheral neuropathies are characterised by frequently occurring sensory neuropathies, such as dysesthesia, numbness, pain and thermohyperesthesia in the feet and hands[1–3], and usually mild motor neuropathies such as muscle weakness and reduction of motor skills including buttoning a shirt.[3] The incidence of paclitaxel-induced neuropathy depends on risk factors including dose per cycle, treatment schedule, duration of infusion and cumulative dose.[3] Amifostine, glutamine, acetyl L-carnitine, BNP7787 and vitamin E have been clinically examined against the paclitaxel-induced neuropathy.[4–8] Although these drugs partially reduced symptoms of neuropathy, they are not commonly used in the clinical setting because of low effectiveness. Thus, new agents strongly reducing the symptoms of neuropathy are required.

Many factors have been reported to be attributed to the development of paclitaxel-induced neuropathy *in vivo*. Those include the generation of radicals[9], the abnormality of Ca^{2+}

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homeostasis[10], the expression of Ca^{2+} channel alpha 2 delta type 1[11,12] and transient receptor potential vanilloid 4 (TRPV4)[13], abnormality in axonal mitochondria of sensory nerves[14], and the activation of microglia in the spinal cord[15] and immunocytes in peripheral nerves.[15–17] In addition, in the sensory nerves of patients with taxane-induced neuropathy, the axonal degeneration decreases in the myelinated fibre density and the loss of large fibres has been exhibited.[3,18–20] However, a detailed mechanism for the development of paclitaxel-induced neuropathy is still largely unknown.

Neurotropin is a non-protein extract derived from the inflamed skin of rabbits inoculated with vaccinia virus. Neurotropin is clinically used to treat various chronic pain conditions, including post herpetic neuralgia, lower back pain, cervicodynia and peripheral neuropathies, and hyperesthesia of subacute myelo-optic neuropathy (SMON). Although neurotropin is available in Japan and some other countries, the National Institute of Nursing Research (NINR) in the United States is now examining the safety and effectiveness of neurotropin for preventing or easing pain associated with fibromyalgia and treating chronic pain after injury to a limb or a large nerve. However, the effect of neurotropin on the paclitaxel-induced neuropathy remains unexplored. Accordingly, we examined the effect of neurotropin on the paclitaxel-induced neuropathy in rat behavioural models. We also examined the effect of neurotropin on the axonal degeneration, considered to be one of the mechanisms for the paclitaxel-induced neuropathy, in PC12 (a neuroendocrine rat pheochromocytoma), dorsal root ganglion (DRG) cells, and in rat sciatic nerve. Furthermore, we investigated the effect of neurotropin on the anti-tumour activity of paclitaxel.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats weighing 200–250 g (Kyudo Co., Saga, Japan) were used for the paclitaxel-induced peripheral neuropathy model. Male C57BL/6 mice weighing 15–20 g (Japan SLC inc., Fukuoka, Japan) were used for the *in vivo* tumour growth model. Animals were housed in groups of four to five per cage, with lights on from 0800 to 2000 h. Animals had free access to food and water in their home cages. All experiments were approved by the Experimental Animal Care and Use Committee of Kyushu University according to the National Institutes of Health guidelines.

2.2. Drugs

Paclitaxel (Taxol®; 6 mg/ml in Cremophor EL/ethanol 1:1) was obtained from Bristol-Myers Squibb (Tokyo, Japan). Neurotropin was a generous gift from Nippon Zoki Pharmaceutical Co. (Osaka, Japan). In the paclitaxel-induced peripheral neuropathy model, paclitaxel (6 mg/kg) or vehicle (50% Cremophor EL/ethanol) was injected i.p. once a week for 4 weeks (Days 0, 7, 14, and 21). Neurotropin [200 Neurotropin Unit (NU)/kg] was injected p.o. three times a week for 4 weeks (Days 0, 1, 2, 7, 8, 9, 14, 15, 16, 21, 22, and 23). The dose of neurotropin was chosen based on a previous report.[21] The volume of vehicle or drug solution injected was 10 ml/kg for all drugs. Behavioural testing was performed blind with respect to drug administration.

2.3. Von Frey test for mechanical allodynia

The Von Frey test was performed before the first drug administration (on Day 0) and on Days 10 and 24. 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 2–15 g bending force were applied to the mid-plantar skin of each hind paw six times, with each application held for 6 s. To determine 50% paw withdrawal thresholds, withdrawal responses to the stimulation of Von Frey filaments were counted.

2.4. Acetone test for cold hyperalgesia

The acetone test was performed before the first drug administration (on Day 0) and on Day 24 according to the method described by Flatters and Bennett.[22] 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 rats were observed for 40 s from the start of the acetone spraying. To determine latencies, the time was recorded from starting spray up to the occurrence of the first avoidance response.

2.5. Grip strength test for motor strength

The grip strength test was performed before the first drug administration (on Day 0) and on Day 25. The grip strength test was performed with the tension gauge (Oba Keiki Co. Ltd., Tokyo, Japan) and by consulting the method described by Authier and colleagues.[23] Rats were placed with both forepaws inside the front grip grid. When a rat gripped the grid, it was steadily pulled backwards by the tail until its grip was broken. The reading on the strain gauge was recorded four times and the mean value was used.

2.6. Balance beam test for motor coordination

The balance beam test was performed before the first drug administration (on Day 0) and on Day 25. The balance beam test was performed by consulting the method described by Jeljeli and colleagues.[24] Rats were trained to travel from the end of a wooden beam (80 × 40 × 4 cm) into a darkened goal box (29 × 25 × 10 cm). On the testing day, rats were placed on the end of the beam and the distances travelled by the rats were measured three times and the mean value was used.

2.7. Cell lines and cultures

PC12 and rat breast carcinoma Walker 256 cells were obtained from the American Type Culture Collection (Walkersville, MD, USA). Human lung carcinoma A549 cells were obtained from

the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). Lewis lung carcinoma (LLC) cells were obtained from RIKEN (Saitama, Japan). L 4-5 DRG cells were removed from male Sprague–Dawley rats (6 weeks old), which anaesthetise with sodium pentobarbital, and primary cultured. Ganglia was incubated with 0.125% (w/v) collagenase type 1 (Worthington Biochemical Corp, NJ, USA) at 37 °C for 90 min followed by incubation with 0.25% (w/v) trypsin-EDTA (Gibco BRL, USA) for 30 min. PC12 cells were grown in RPMI 1640 medium (MP Biomedicals Inc., Irvine, CA, USA) supplemented with 2 mM L-glutamine, 10% horse serum, and 5% FBS. DRG and LLC cells were grown in Dulbecco's modified Eagle's medium (MP Biomedicals Inc.) with 2 mM L-glutamine and 10% FBS. Walker 256 cells were grown in 199 medium (MP Biomedicals Inc.) with 2 mM L-glutamine and 5% horse serum. A549 cells were grown in RPMI 1640 medium with 2 mM L-glutamine and 10% FBS. All cell lines were cultured on 80 cm^2 tissue culture flasks (Nunc Apogent Co., Roskilde, Denmark) at 37 °C in air supplemented with 5% CO_2 under humidified conditions.

2.8. *Assay of PC12 and DRG neurite outgrowths*

PC12 cells were seeded at a density of 1×10^4 cells/cm^2 onto 24 well plates (Falcon, Becton Dickinson Co. Ltd., Franklin Lakes, NJ, USA) and were used for experiments on the following day. Neurite outgrowth in PC12 cells was induced by 10 μM forskolin (Carbiochem, EMD Chemicals Inc., Darmstadt, Germany) at 3 h before paclitaxel and neurotropin exposures. DRG cells were seeded onto 24 well plates and were cultured for a week so that neurites were extended. Both cell types were exposed to paclitaxel (10 ng/ml) and neurotropin (0, 0.001, 0.003, 0.01, or 0.03 NU/ml) for 24 or 168 h. After incubation with paclitaxel and neurotropin, dead cells were stained with trypan blue (Gibco BRL, Grand Island, NY, USA). Cells were monitored by a phase contrast microscope and neurite lengths in living cells were measured by analysis software (Image J 1.36; Wayne Rasband, National Institutes of Health, MD, USA). We also measured the LDH leakage from the PC12 cell to investigate whether the exposure to drugs for 168 h induced cell injury or not. The LDH leakage was expressed as the percentage of LDH released into medium to total. LDH activity was determined using LDH assay kit (Takara Biochemicals, Osaka, Japan).

2.9. *Toluidine blue staining for sciatic nerve*

On Day 25, sciatic nerves were harvested from rats anaesthetised with sodium pentobarbital. Nerves were fixed in 2% (w/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.4, 4 °C) for 4 h followed by washing with 0.1 M phosphate buffer. After 8% (w/v) sucrose-substitution, samples were embedded in Epon. Each section was stained with toluidine blue. Sample sections were evaluated using light microscopy.

2.10. *Immunostaining of beta tubulin for imaging of microtubule aggregates*

Immunofluorescent staining for beta tubulin was carried out using a rabbit monoclonal antibody (Cell Signaling Technology, Inc., Beverly, MA, USA). Briefly, cells were cultured on cover slips at the density of 1×10^4 cells/cm^2 and incubated for 24 h. The cover slips were then rinsed with ice-cold phosphate-buffered saline and fixed with 3% (w/v) ice-cold paraformaldehyde for 30 min at −20 °C. The beta tubulin antibody was diluted (1:100) with phosphate-buffered saline containing 5% (w/v) bovine serum albumin and 0.1% Triton X-100. Cells were incubated with diluted antibody solution overnight in a humidified chamber at 4 °C. After washing with phosphate-buffered saline, cover slips were incubated at room temperature for 2 h with goat anti-rabbit IgG (1:100 dilution in phosphate-buffered saline) that was labelled fluorescein isothiocyanate (FITC). Beta tubulin was visualised by the fluorescence microscope.

2.11. *Tumour cytotoxicity assay*

A549 and Walker 256 cells were seeded at a density of 2×10^4 cells/cm^2 onto 24 well plates and were used for experiments on the following day. Cells were exposed to paclitaxel (10 ng/ml) and neurotropin (0, 0.001, 0.003, 0.01, or 0.03 NU/ml) for 6, 12, 24, 48, or 72 h. The cell viability was assessed by the mitochondrial activity in reducing WST-8 (2-(2-meth-

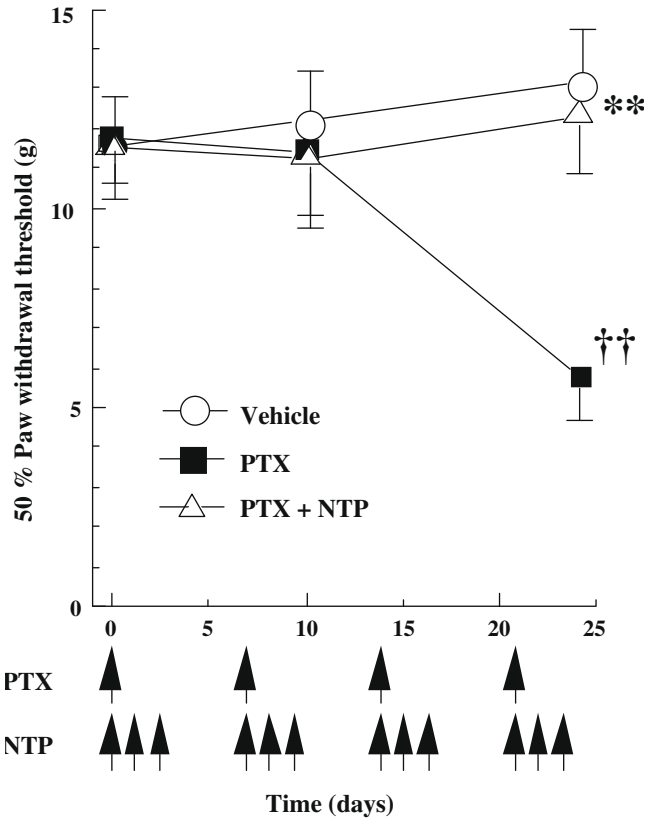

Fig. 1 – Mechanical allodynia in the Von Frey test in rats. Rats were treated with paclitaxel (PTX, 6 mg/kg, i.p.) once a week for 4 weeks. Neurotropin (NTP, 200 NU/kg) was administered orally three times a week for 4 weeks. Results are expressed as mean ± SEM of 7–8 animals on Days 0, 10, and 24. ††P<0.01 compared with vehicle, **P<0.01 compared with PTX alone.

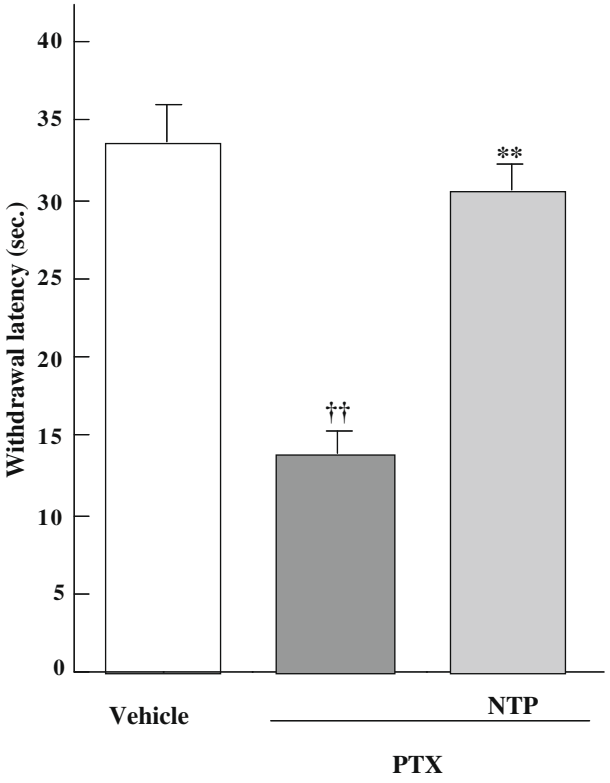

Fig. 2 – Cold hyperalgesia in the acetone test in rats. Rats were treated with paclitaxel (PTX, 6 mg/kg, i.p.) once a week for 4 weeks. Neurotropin (NTP, 200 NU/kg) was administered orally three times a week for 4 weeks. Results are expressed as mean ± SEM of 7–9 animals. ††P<0.01 compared with vehicle, **P<0.01 compared with PTX alone.

oxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2 H-tetrazolium, monosodium salt) to formazan. At 6, 12, 24, 48, or 72 h after incubation with paclitaxel and neurotropin, the cells were washed with phosphate-buffered saline, then 210 μl of serum-free medium and 10 μl of WST-8 assay solution (Cell Counting Kit-8; Dojindo Laboratory, Kumamoto, Japan) were added and incubated for 1 h at 37 °C in humidified air supplemented with 5% CO_2. The incubation medium was carefully taken and transferred to 96 well flat-bottom plastic plates (Corning Costar, Corning, NY, USA). The amount of formed formazan dye was measured from the absorbance at 450 nm with a reference wavelength of 620 nm using a microplate reader (Immuno-mini NJ-2300; Inter Medical, Tokyo, Japan).

2.12. *Tumour growth analysis using mouse model*

LLC cells (1.5×10^6 cells per mouse in 50 μl serum free medium) were implanted subcutaneously in the chests of C57BL/6 mice. Three days after implantation of tumour cells, administration of drugs was started. Paclitaxel (6 mg/kg, i.p.) and neurotropin (600 NU/kg, p.o.] were injected once a day for 12 days. The tumour volumes were calculated as follows:Volume (mm^3) = Length (mm) × Width $(mm)^2$.

2.13. *Statistical analyses*

Values were expressed as the mean ± SEM. The values were analysed with a one-way analysis of variance (ANOVA) followed by the Tukey–Kramer test (StatView; Abacus Concepts, Berkely, CA, USA) to determine differences among the groups. The values of tumour cytotoxicity were expressed as percent-

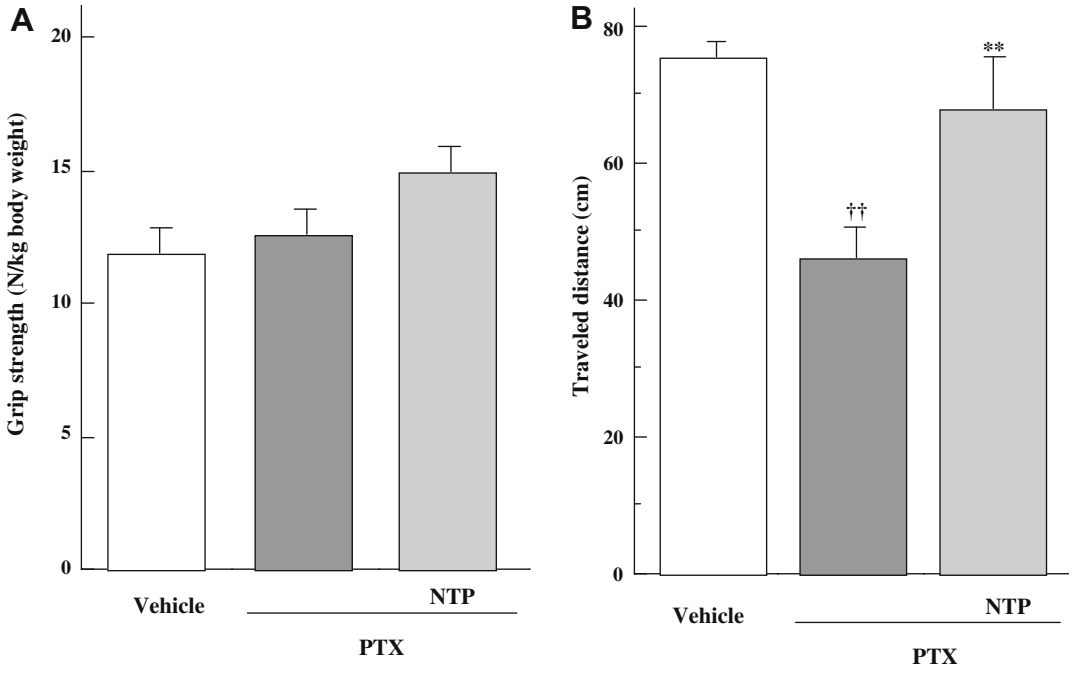

Fig. 3 – Motor functions in the grip strength (A) and balance beam (B) tests. Rats were treated with paclitaxel (PTX, 6 mg/kg, i.p.) once a week for 4 weeks. Neurotropin (NTP, 200 NU/kg) was administered orally three times a week for 4 weeks. Results are expressed as mean ± SEM of 8–9 animals. ††P<0.01 compared with vehicle, **P<0.01 compared with PTX alone.

ages of level of vehicle-treated group. The values of tumour cytotoxity and tumour volumes were analysed by two-way (repeated-measures) ANOVA. A probability level of $P<0.05$ was accepted as statistically significant.

3. Results

In a preliminary test, we examined the effect of paclitaxel at various doses (3, 6, 10 and 15 mg/kg, i.p.) on the mechanical

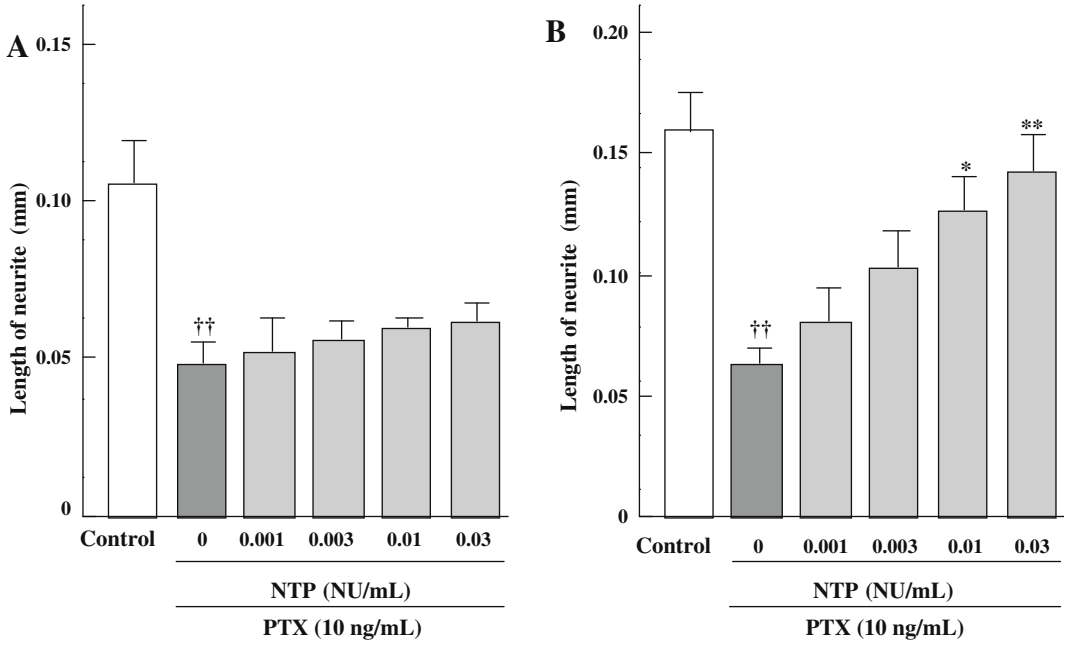

Fig. 4 – Effect of neurotropin (NTP) on neurite degeneration induced by paclitaxel (PTX) in PC12 cells. PC12 cells were incubated with PTX (10 ng/ml) for 24 h (A) or 168 h (B) in the presence or absence of various concentrations of NTP. The neurite lengths were measured using image analysis software (Image J 1.36). Results are expressed as mean ± SEM ($n = 4$). ††$P<0.01$ compared with control, *$P<0.05$, **$P<0.01$ compared with PTX alone.

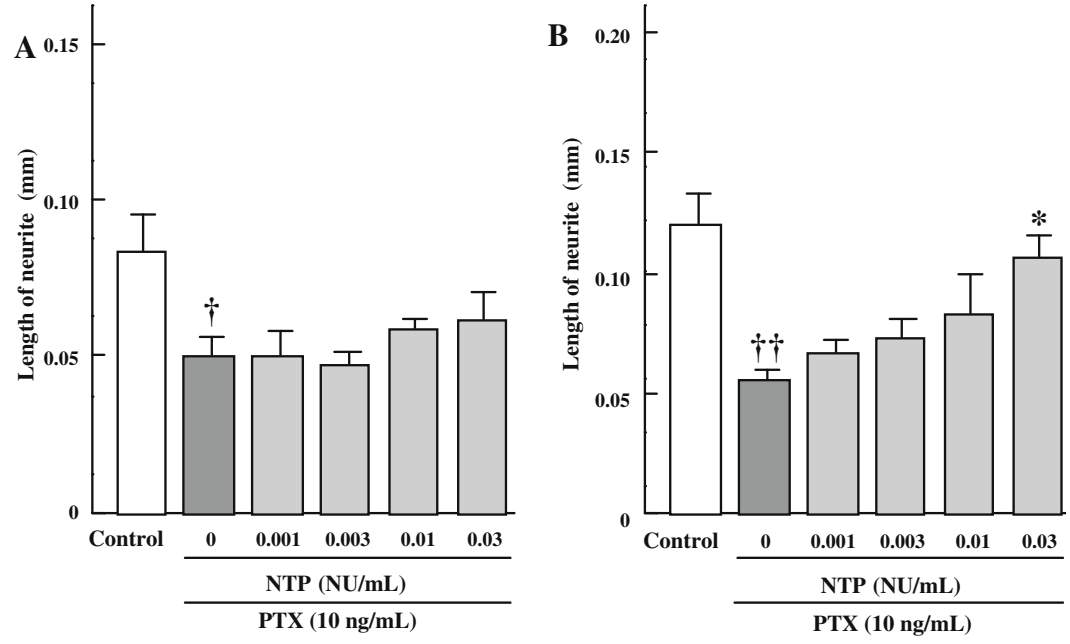

Fig. 5 – Effect of neurotropin (NTP) on neurite degeneration induced by paclitaxel (PTX) in rat DRG neurons. Primary cultured DRG neurons were incubated with PTX (10 ng/ml) for 24 h (A) or 168 h (B) in the presence or absence of various concentrations of NTP. The neurite lengths were measured using image analysis software (Image J 1.36). Results are expressed as mean ± SEM ($n = 4$). †$P<0.05$, ††$P<0.01$ compared with control, *$P<0.05$ compared with PTX alone.

allodynia in the Von Frey test. Rats treated with higher doses (10 and 15 mg/kg) died at the rate from 43 to 63%. On the other hand, paclitaxel at the dose of 3 mg/kg had no effect on the mechanical allodynia. Therefore, we selected 6 mg/kg as the appropriate dosage of paclitaxel.

In the present study, rats were treated with vehicle, paclitaxel (6 mg/kg, i.p.) alone or paclitaxel (6 mg/kg, i.p.) and neurotropin (200 NU/kg, p.o.) for 4 weeks. The mortality rate of each group was 9 (1/11), 10 (1/10) and 10 (1/10)%. No deterioration in general status was observed. In addition, there was no difference in change of body weight among three groups (data not shown).

3.1. *Effect of neurotropin on mechanical allodynia in the Von Frey test in paclitaxel-treated rats*

Before the first drug administration, each group had an equivalent 50% withdrawal threshold in the Von Frey test. Paclitaxel (6 mg/kg, i.p.) significantly reduced the 50% paw withdrawal threshold compared with vehicle on Day 24 [$F(2,20) = 9.824$, $P<0.01$ by one-way ANOVA; $P<0.01$ by Tukey–Kramer test, Fig. 1]. Neurotropin (200 NU/kg, p.o.) reversed the reduction of 50% paw withdrawal threshold by paclitaxel almost completely ($P<0.01$ by Tukey–Kramer test).

3.2. *Effect of neurotropin on cold hyperalgesia in the acetone test in paclitaxel-treated rats*

Before the first drug administration, each group had equivalent withdrawal latencies in the acetone test (data not shown). Paclitaxel (6 mg/kg, i.p.) significantly shortened the withdrawal latency compared with vehicle in the acetone test [$F(2,22) = 32.869$, $P<0.0001$ by one-way ANOVA; $P<0.01$ by Tukey–Kramer test, Fig. 2]. Neurotropin (200 NU/kg, p.o.) reversed the paclitaxel-induced shortening of the withdrawal latency by 85% ($P<0.01$ by Tukey–Kramer test).

3.3. *Effect of neurotropin on motor function in the grip strength and balance beam tests in paclitaxel-treated rats*

Before the first drug administration, each group had equivalent values in the grip strength test and travelled distance in the balance beam test (data not shown). In the grip strength test, there was no significant difference among three groups [$F(2,22) = 2.354$, $P>0.1$ by one-way ANOVA, Fig. 3]. In the balance beam test, paclitaxel (6 mg/kg, i.p.) significantly decreased the travelled distance compared with vehicle [$F(2,22) = 12.046$, $P<0.001$ by one-way ANOVA; $P<0.01$ by Tukey–Kramer test, Fig. 3B]. Neurotropin (200 NU/kg, p.o.) reversed the paclitaxel-induced decrease of the travelled distance by 75% ($P<0.01$ by Tukey–Kramer test).

3.4. *Effect of neurotropin on paclitaxel-induced neurite degeneration in PC12 cells*

The exposure to paclitaxel (10 ng/ml) for 24 h significantly shortened the length of neurites in cultured PC12 cells [$F(5,18) = 6.413$, $P<0.01$ by one-way ANOVA; $P<0.01$ by Tukey–Kramer test, Fig. 4A]. The co-exposure to neurotropin (0.001–0.03 NU/ml) for 168 h significantly extended the length of neurites compared with the paclitaxel-treated group in the concentration-dependent manner [$F(5,18) = 7.448$, $P<0.001$ by

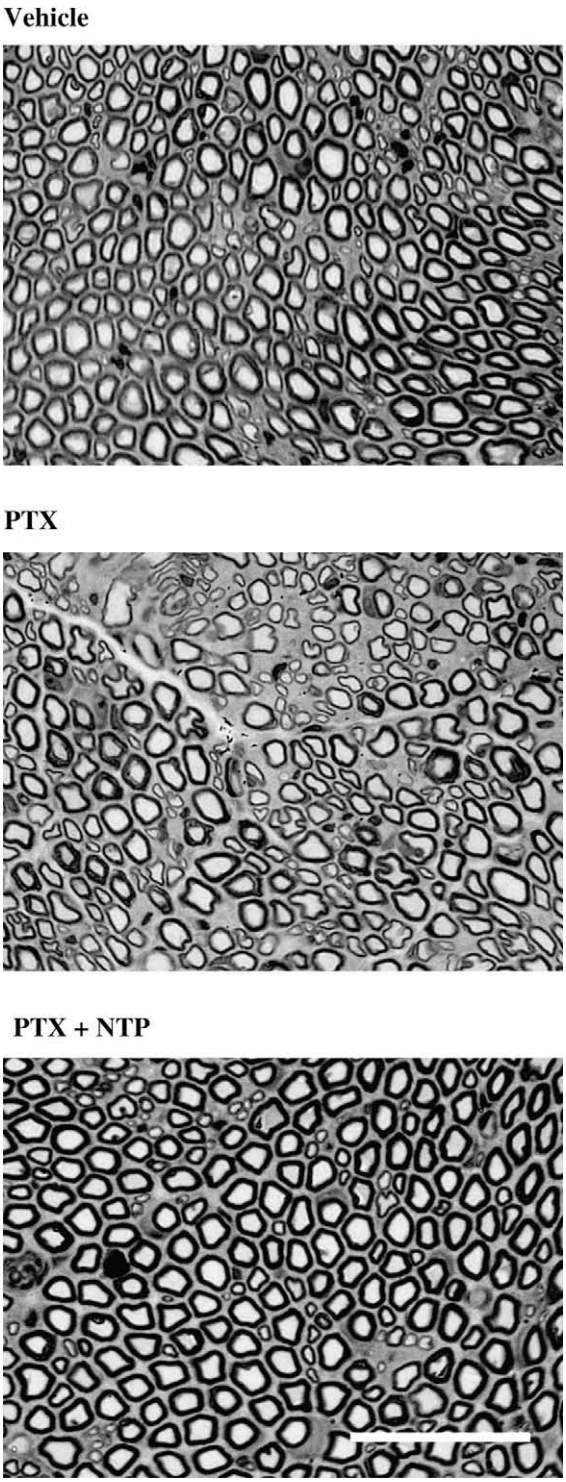

Fig. 6 – Effect of neurotropin (NTP) on histological change induced by paclitaxel (PTX) in rat sciatic nerve. Rats were treated with paclitaxel (PTX, 6 mg/kg, i.p.) once a week for 4 weeks. Neurotropin (NTP, 200 NU/kg) was administered orally three times a week for 4 weeks. On Day 25, the sciatic nerve was harvested, and samples were stained with toluidine blue. Photographs were originally magnified 800 ×. Scale bar 50 μm.

one-way ANOVA; 0.01 NU/ml; $P<0.05$, 0.03 NU/ml; $P<0.01$ by Tukey–Kramer test, Fig. 4B], although the 24-h co-exposure to neurotropin did not significantly affect it. In addition, the exposure to paclitaxel or co-exposure to neurotropin for 168 h did not significantly increase the LDH leakage compared with control group (data not shown).

3.5. *Effect of neurotropin on paclitaxel-induced neurite degeneration in rat DRG neurons*

The exposure to paclitaxel (10 ng/ml) for 24 h significantly shortened the length of neurites in rat DRG neurons, but the co-exposure to neurotropin (0.001–0.03 NU/ml) did not affect this change [$F(5,18) = 3.432$, $P<0.05$ by one-way ANOVA; $P<0.05$ by Tukey–Kramer test, Fig. 5A]. When co-exposed for 168 h, neurotropin (0.03 NU/ml) significantly extended the length of neurites compared with the paclitaxel-treated group [$F(5,18) = 6.024$, $P<0.01$ by one-way ANOVA; $P<0.05$ by Tukey–Kramer test, Fig. 5B].

3.6. *Effect of neurotropin on paclitaxel-induced histolog ical change in rat sciatic nerve*

No histological abnormalities in sciatic nerve were observed in vehicle-treated rats. Paclitaxel (6 mg/kg, i.p.) induced the decrease in the density of myelinated fibres and the degeneration of myelinated fibres in rat sciatic nerve. These histological changes were not observed in the tissue of rat treated with co-administration of paclitaxel and neurotropin (see Fig. 6).

3.7. *Effect of neurotropin on paclitaxel-induced microtu bule aggregates*

The exposure of cultured Walker 256 or A549 cells to paclitaxel (10 ng/ml) for 24 h caused beta tubulin aggregates (Fig. 7, arrows). These beta tubulin aggregates were not affected by neurotropin (0.03 NU/ml) in two kinds of cell.

3.8. *Effect of neurotropin on the tumour cytotoxicity of paclitaxel*

The exposure of cultured Walker 256 or A549 cells to paclitaxel (10 ng/ml) for 6, 12, 24, 48, or 72 h caused time-dependent decreases in tumour cell viability as assessed by mitochondrial enzyme activity using the WST-8 assay (Fig. 8). In Walker 256 cells, repeated-measures ANOVA revealed a significant time effect [$F(4,60) = 654.757$, $P<0.0001$], but a non-significant drug effect [$F(4,15) = 0.855$] or drug × time interaction [$F(16,60) = 1.508$]. In A549 cells, repeated-measures ANOVA also revealed a significant time effect [$F(4,60) = 115.535$, $P<0.0001$], but a non-significant drug effect [$F(4,15) = 1.222$] or drug × time interaction [$F(16,60) = 1.007$]. Therefore, neurotropin (0.001-0.03 NU/ml) had no effect on the paclitaxel-induced decrease of tumour cell viability in either cell line.

3.9. *Effect of neurotropin on the anti-tumour activity of paclitaxel in tumour cells-implanted mice*

Paclitaxel (6 mg/kg, i.p.) inhibited the increase of tumour volumes in tumour cells-implanted mice (Fig. 9). Repeated-measures ANOVA revealed a significant drug effect [$F(2,25) = 7.685$, $P<0.01$], a significant time effect [$F(5,125) = 114.267$, $P<0.0001$] and drug × time interaction [$F(10,125) = 7.004$, $P<0.0001$]. Paclitaxel (6 mg/kg, i.p.) significantly inhibited the increase of tumour volumes compared with vehicle on Day 18 [$F(2,23) = 7.063$, $P<0.01$ by one-way ANOVA; $P<0.05$ by Tukey–Kramer test]. Neurotropin (600 NU/kg, p.o.) had no effect on the paclitaxel-induced inhibition of tumour growth.

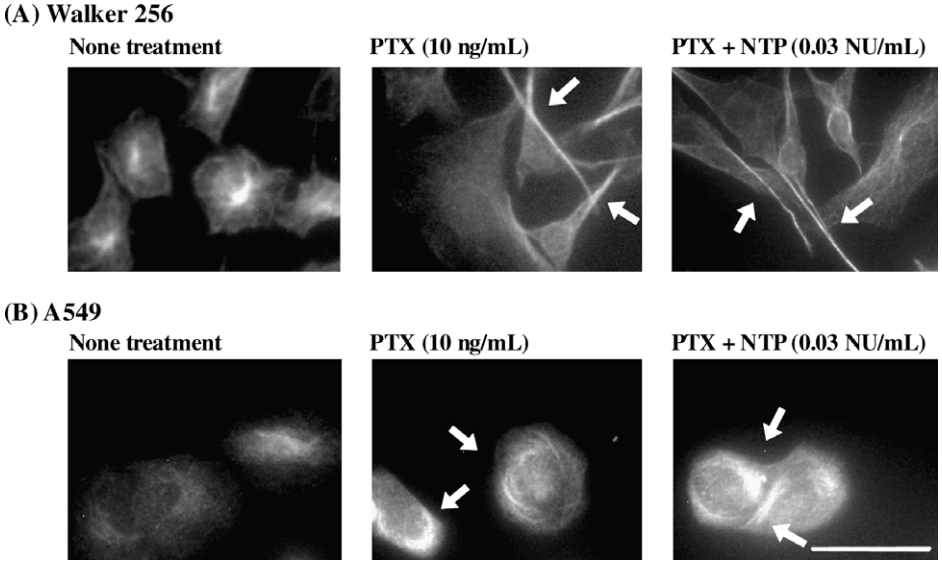

Fig. 7 – Effect of neurotropin (NTP) on microtubule aggregates induced by paclitaxel (PTX). Walker 256 (A) and A549 cells (B) were incubated with PTX (10 ng/ml) for 24 h in the presence or absence of NTP (0.03 NU/ml). Cells were immunostained with beta tubulin for imaging of microtubule aggregates. The arrows indicate beta tubulin. Photographs were originally magnified 400 ×. Scale bar, 100 μm.

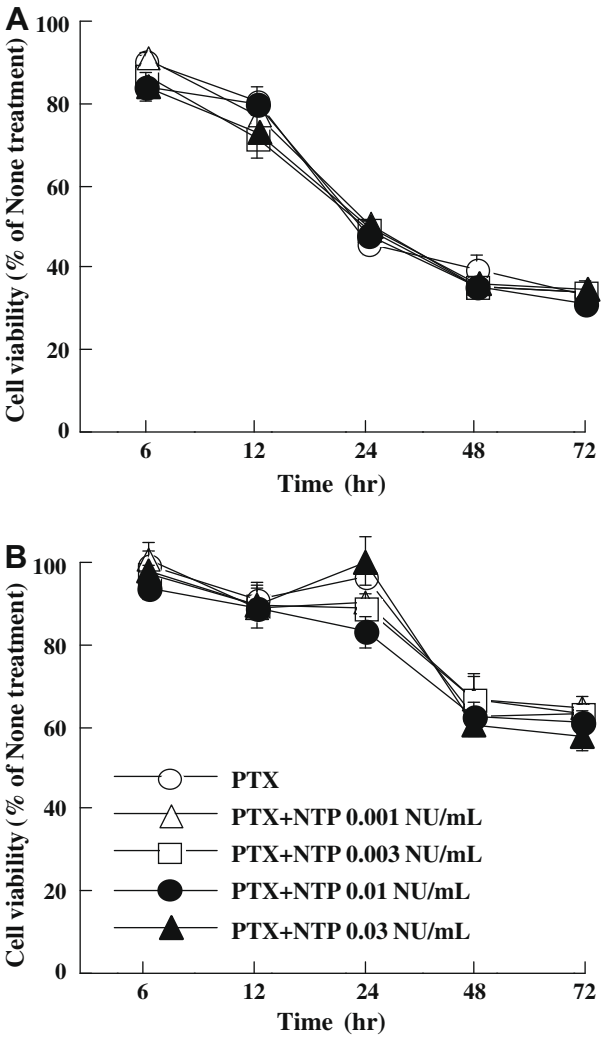

Fig. 8 – Effect of neurotropin (NTP) on the tumour cytotoxicity of paclitaxel (PTX). Walker 256 (A) and A549 cells (B) were incubated with PTX (10 ng/ml) for 6, 12, 24, 48, or 72 h in the presence or absence of various concentrations of NTP. Cell viability was measured by WST-8 assay. Results are expressed as percentages of the viability of the vehicle-treated group (n = 4).

4. Discussion

In the present study, neurotropin reversed paclitaxel-induced reduction of threshold in the Von Frey test almost completely and there was a shortening of withdrawal latency in the acetone test by 85%. Recently, acetyl-L-carnitine or gabapentin has been reported to reduce the paclitaxel-induced allodynia and hyperalgesia in rats by 34–70%.[12,25] Acetyl-L-carnitine is present throughout the central and peripheral nervous systems and plays an essential role in the oxidation of free fatty acids.[26] Acetyl-L-carnitine ameliorates the paclitaxel-induced neuropathy in clinical trials.[6] Gabapentin is an anticonvulsant drug which binds to the neuronal voltage-gated Ca^{2+} channel alpha 2 delta type 1.[27] Gabapentin has also been reported to ameliorate several neuropathic pains, such as diabetic neuropathy[28,29], cancer pain[30] and post herpetic neuralgia.[31] Our data suggest that neurotropin may be useful for the treatment of paclitaxel-induced neuropathy.

Clinical studies have shown that the axonal degeneration of nerves is caused by paclitaxel, as well as a reduction of myelinated fibre density and the loss of large fibres.[3,18–20] In the present study, we examined the effects of paclitaxel and neurotropin on the neurite outgrowths in PC12 and DRG neurons, and found that neurotropin repaired the paclitaxel-induced axonal degeneration in these neurons. Moreover, we found that neurotropin prevented the paclitaxel-induced axonal degeneration in sciatic nerve. These reparations of axonal degeneration by neurotropin may at least partially contribute to the reversal of the paclitaxel-induced neuropathy.

In the model used in the present study, we observed the mechanical allodynia (Von Frey test) and cold hyperalgesia (acetone test) after paclitaxel administration, consistent with previous reports.[22,23,32,33] We also observed the impairment of motor coordination (balance beam test) after paclitaxel administration consistent with previous results[34,35], with the exception of one result.[33] These discrepancies may be due to the difference in the administration amount, route and interval of paclitaxel. In the grip strength test, paclitaxel has not been shown to reduce the muscle strength, consistent with previous results.[23,34] We also did not observe the deterioration in general status. In addition, there was no difference in change of body weight among the three groups. Hence, it is unlikely that the impairment of motor coordination is due to the muscle weakness, change of body weight and deterioration in general status. Thus, the present model is characterised by both sensory neuropathy (mechanical allodynia and cold hyperalgesia) and motor neuropathy (impairment of motor coordination).

Though we did not measure the mechanical hyperalgesia in this study, single treatment with paclitaxel (32 mg/kg, i.p.) has been reported to induce mechanical hyperalgesia in paw pressure tests in rats.[23] We found that neurotropin re-

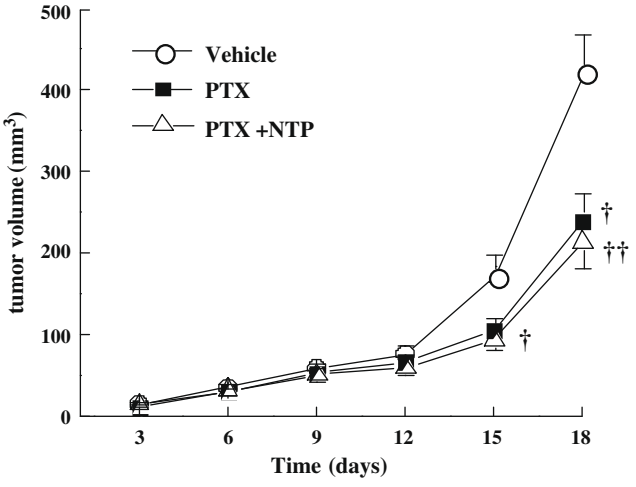

Fig. 9 – Effect of neurotropin (NTP) on the anti-tumour effect of paclitaxel (PTX). The LLC cells-implanted mice were treated with paclitaxel (PTX, 6 mg/kg, i.p.) and neurotropin (NTP, 600 NU/kg, p.o.) once a day for 12 days. Results are expressed as mean ± SEM of 8–10 animals on Days 3, 6, 9, 12, 15 and 18. †P<0.05, ††P<0.01 compared with vehicle.

versed the paclitaxel-induced axonal degeneration. The axonal degeneration is known to underlie neuropathy symptoms including hyperalgesia.[36,37] Therefore, neurotropin might ameliorate paclitaxel-induced mechanical hyperalgesia as well as mechanical allodynia and cold hyperalgesia.

Neurotropin is used clinically based on the following three actions; analgesia, amelioration of paresthesia (cold sensation) and antiallergy property. There have been reports of the involvement of enhancement of noradrenergic and serotonergic systems in the analgesic action of neurotropin[21], and the involvement of modification of abnormal discharge of hypothalamic neurons[38], improvement of peripheral blood flow[39] and modulation of autonomic nerves[40–42] in the amelioration of paresthesia by neurotropin. Therefore, these effects might be partially related to the amelioration of the paclitaxel-induced neuropathy by neurotropin. More recently, neurotropin has been reported to induce expression of thioredoxin, a redox-regulating molecule, in A549 cells.[43] Thioredoxin plays a critical regulatory role in nerve growth factor-mediated signal transduction and neurite outgrowth in PC12 cells.[44] Furthermore, thioredoxin-interacting protein, an endogenous inhibitor of antioxidants, is increased in DRG neurons from diabetic rats.[45] Hyperglycaemia promotes oxidative stress through inhibition of thioredoxin function by thioredoxin-interacting protein.[46] Thioredoxin might be partially related to the reparation of axonal degeneration by neurotropin.

The present results also show that neurotropin does not affect the paclitaxel-induced microtubule aggregates in Walker 256 or A549 cells. Therefore, it is unlikely that neurotropin repairs the paclitaxel-induced axonal degeneration by inhibitory effect on the paclitaxel-induced microtubule aggregates. Additionally, neurotropin had no effect on the paclitaxel-induced tumour cytotoxicity in these tumour cells. Furthermore, neurotropin had no effect on the anti-tumour effect of paclitaxel in tumour cells-implanted mice.

In conclusion, the present study clarifies that neurotropin ameliorates the paclitaxel-induced neuropathy in the rat model and repairs the paclitaxel-induced axonal degeneration, without affecting the anti-tumour activity of paclitaxel. Therefore, neurotropin is expected to be useful as a therapeutic drug for clinical paclitaxel-induced neuropathy.

Conflict of interest statement

None declared.

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REFERENCES

1. Quasthoff S, Hartung HP. Chemotherapy-induced peripheral neuropathy. *J Neurol* 2002;**249**:9–17.
2. Dougherty PM, Cata JP, Cordella JV, Burton A, Weng HR. Taxol-induced sensory disturbance is characterized by preferential impairment of myelinated fiber function in cancer patients. *Pain* 2004;**109**:132–42.
3. Lee JJ, Swain SM. Peripheral neuropathy induced by microtubule-stabilizing agents. *J Clin Oncol* 2006;**24**:1633–42.
4. Lorusso D, Ferrandina G, Greggi S, et al. Multicenter Italian Trials in Ovarian Cancer investigators. Phase III multicenter randomized trial of amifostine as cytoprotectant in first-line chemotherapy in ovarian cancer patients. *Ann Oncol* 2003;**14**:1086–93.
5. Stubblefield MD, Vahdat LT, Balmaceda CM, Troxel AB, Hesdorffer CS, Gooch CL. Glutamine as a neuroprotective agent in highdose paclitaxel-induced peripheral neuropathy: A clinical and electrophysiologic study. *Clin Oncol (R Coll Radiol)* 2005;**17**:271–6.
6. Bianchi G, Vitali G, Caraceni A, et al. Symptomatic and neurophysiological responses of paclitaxel- or cisplatin-induced neuropathy to oral acetyl-L-carnitine. *Eur J Cancer* 2005;**41**:1746–50.
7. Takeda K, Negoro S, Matsui K. Phase I safety and pharmacokinetic trial of BNP7787 in patients receiving cisplatin (CDDP) and paclitaxel (PTX) for advanced non-small cell lung cancer (NSCLC): An Osaka phase I study group trial. *Proc Am Soc Clin Oncol* 2002;**21**:114.
8. Argyriou AA, Chroni E, Koutras A, et al. Vitamin E for prophylaxis against chemotherapy-induced neuropathy: A randomized controlled trial. *Neurology* 2005;**64**:26–31.
9. Bárdos G, Móricz K, Jaszlits L, et al. BGP-15, a hydroximic acid derivative, protects against cisplatin or taxol-induced peripheral neuropathy in rats. *Toxicol Appl Pharmacol* 2003;**190**:9–16.
10. Siau C, Bennett GJ. Dysregulation of cellular calcium homeostasis in chemotherapy-evoked painful peripheral neuropathy. *Anesth Analg* 2006;**102**:1485–90.
11. Matsumoto M, Inoue M, Hald A, Xie W, Ueda H. Inhibition of paclitaxel-induced A-fiber hypersensitization by gabapentin. *J Pharmacol Exp Ther* 2006;**318**:735–40.
12. Xiao W, Boroujerdi A, Bennett GJ, Luo ZD. Chemotherapy-evoked painful Peripheral neuropathy: analagesic effects of gabapentin and effects on expression of the alpha-2-delta type-1 calcium channel subunit. *Neuroscience* 2007;**144**: 714–20.
13. Alessandri-Haber N, Dina OA, Yeh JJ, Parada CA, Reichling DB, Levine JD. Transient receptor potential vanilloid 4 is essential in chemotherapy-induced neuropathic pain in the rat. *J Neurosci* 2004;**24**:4444–52.
14. Flatters SJL, Bennett GJ. Studies of peripheral sensory nerves in paclitaxel-induced painful peripheral neuropathy: evidence for mitochondrial dysfunction. *Pain* 2006;**122**:245–57.
15. Peters CM, Jimenez-Andrade JM, Jonas BM, et al. Intravenous paclitaxel administration in the rat induces a peripheral sensory neuropathy characterized by macrophage infiltration and injury to sensory neurons and their supporting cells. *Exp Neurol* 2006;**203**:42–54.
16. Ledeboer A, Jekich BM, Sloane EM, et al. Intrathecal interleukin-10 gene therapy attenuates paclitaxel-induced mechanical allodynia and proinflammatory cytokine expression in dorsal root ganglia in rats. *Brain Behav Immun* 2006;**21**:686–98.
17. Siau C, Xiao W, Bennett GJ. Paclitaxel- and vincristine-evoked painful peripheral neuropathies: Loss of epidermal innervation and activation of Langerhans cells. *Exp Neurol* 2006;**201**:507–14.
18. Sahenk Z, Barohn R, New P, Mendell JR. Taxol neuropathy: Electrodiagnostic and sural nerve biopsy findings. *Arch Neurol* 1994;**51**:726–9.

19. New PZ, Jackson CE, Rinaldi D, Burris H, Barohn RJ. Peripheral neuropathy secondary to docetaxel (Taxotere). *Neurology* 1996;**46**:108–11.
20. Fazio R, Quattrini A, Bolognesi A, et al. Docetaxel neuropathy: a distal axonopathy. *Acta Neuropathol (Berl)* 1999;**98**:651–3.
21. Kawamura M, Ohara H, Go K, Koga Y, Ienaga K. Neurotropin induces antinociceptive effect by enhancing descending pain inhibitory systems involving 5-HT_3 and noradrenergic α_2 receptors in spinal dorsal horn. *Life Sci* 1998;**62**:2181–90.
22. Flatters SJL, Bennett GJ. Ethosuximide reverses paclitaxel- and vincristine-induced painful peripheral neuropathy. *Pain* 2004;**109**:150–61.
23. Authier N, Gillet JP, Fialip J, Eschalier A, Coudore F. Description of a short-term Taxol®-induced nociceptive neuropathy in rats. *Brain Res* 2000;**887**:239–49.
24. Jeljeli M, Strazielle C, Caston J, Lalonde R. Effects of centrolateral or medial thalamic lesions on motor coordination and spatial orientation in rats. *Neurosci Res* 2000;**38**:155–64.
25. Flatters SJL, Xiao WH, Bennett GJ. Acetyl-L-carnitine prevents and reduces paclitaxel-induced painful peripheral neuropathy. *Neurosci Lett* 2006;**397**:219–23.
26. Fritz IB, Yue KT. Long-chain carnitine acyltransferase and the role of acylcarnitine derivatives in the catalytic increase of fatty acid oxidation induced by carnitine. *J Lipid Res* 1963;**58**:279–88.
27. Gee NS, Brown JP, Dissanayake VU, Offord J, Thurlow R, Woodruff GN. The novel anticonvulsant drug, gabapentin (Neurontin), binds to the alpha2delta subunit of a calcium channel. *J Biol Chem* 1996;**271**:5768–76.
28. Backonja M, Beydoun A, Edwards KR, et al. Gabapentin for the symptomatic treatment of painful neuropathy in patients with diabetes mellitus: a randomized controlled trial. *JAMA* 1998;**280**:1831–6.
29. Gorson KC, Schott C, Herman R, Ropper AH, Rand WM. Gabapentin in the treatment of painful diabetic neuropathy: a placebo controlled, double blind, crossover trial. *J Neurol Neurosurg Psychiatry* 1999;**66**:251–2.
30. Caraceni A, Zecca E, Bonezzi C, et al. Gabapentin for neuropathic cancer pain: a randomized controlled trial from the Gabapentin Cancer Pain Study Group. *J Clin Oncol* 2004;**22**:2909–17.
31. Rowbotham M, Harden N, Stacey B, Bernstein P, Magnus-Miller L. Gabapentin for the treatment of postherpetic neuralgia: a randomized controlled trial. *JAMA* 1998;**280**:1837–42.
32. Dina OA, Chen X, Reichling D, Levine JD. Role of protein kinase Cε and protein kinase A in a model of paclitaxel-induced painful peripheral neuropathy in the rat. *Neuroscience* 2001;**108**:507–15.
33. Polomano RC, Mannes AJ, Clark US, Bennett GJ. A painful peripheral neuropathy in the rat produced by the chemotherapeutic drug, paclitaxel. *Pain* 2001;**94**:293–304.
34. Cliffer KD, Siuciak JA, Carson SR, et al. Physiological characterization of taxol-induced large-fiber sensory neuropathy in the rat. *Ann Neurol* 1998;**43**:46–55.
35. Wang MS, Davis AA, Culver DG, Wang Q, Powers JC, Glass JD. Calpain inhibition protects against Taxol-induced sensory neuropathy. *Brain* 2004;**127**:671–9.
36. Wagner R, Heckman HM, Myers RR. Wallerian degeneration and hyperalgesia after peripheral nerve injury are glutathione-dependent. *Pain* 1998;**77**:173–9.
37. Wang MS, Davis AA, Culver DG, Glass JD. WldS mice are resistant to paclitaxel (taxol) neuropathy. *Ann Neurol* 2002;**52**:442–7.
38. Nakamura K, Ono T, Nishijo H, Tamura R. Action of neurotropin on rat hypothalamic neurons in tissue slices. *Brain Res Bull* 1990;**24**:811–7.
39. Hata T, Kita T, Kawabata A, Itoh E, Nishimura Y. Changes of tissue blood flow in mice loaded with SART (repeated cold) stress or restraint and water immersion stress and the effect of administered neurotropin. *Jpn J Pharmacol* 1986;**41**:69–79.
40. Yoneda R, Kita T, Hata T, Namimatsu A. Experimental partial sympathicotonia, and effects of some drugs on it in restraint and water immersion stressed animals. *J Pharmacobiodyn* 1980;**3**:692–701.
41. Hata T, Kita T, Higashiguchi T, Ichida S. Total acetylcholine content, and activities of choline acetyltransferase and acetylcholinesterase in brain and duodenum of SART-stressed (repeated cold-stressed) rat. *Jpn J Pharmacol* 1986;**41**:475–85.
42. Tanaka M, Ida Y, Tsuda A, Tsujimaru S. Effects of neurotropin on regional brain noradrenaline metabolism in rats. *Jpn J Pharmacol* 1989;**49**:187.
43. Hoshino Y, Nakamura T, Sato A, Mishima M, Yodoi J, Nakamura H. Neurotropin demonstrates cytoprotective effects in lung cells through the induction of thioredoxin-1. *Am J Respir Cell Mol Biol* 2007;**37**:438–46.
44. Bai J, Nakamura H, Kwon YW, et al. Critical roles of thioredoxin in nerve growth factor-mediated signal transduction and neurite outgrowth in PC12 cells. *J Neurosci* 2003;**23**:503–9.
45. Price SA, Gardiner NJ, Duran-Jimenez B, Zeef LA, Obrosova IG, Tomlinson DR. Thioredoxin interacting protein is increased in sensory neurons in experimental diabetes. *Brain Res* 2006;**1116**:206–14.
46. Schulze PC, Yoshioka J, Takahashi T, He Z, King GL, Lee RT. Hyperglycemia promotes oxidative stress through inhibition of thioredoxin function by thioredoxin-interacting protein. *J Biol Chem* 2004;**279**:30369–74.