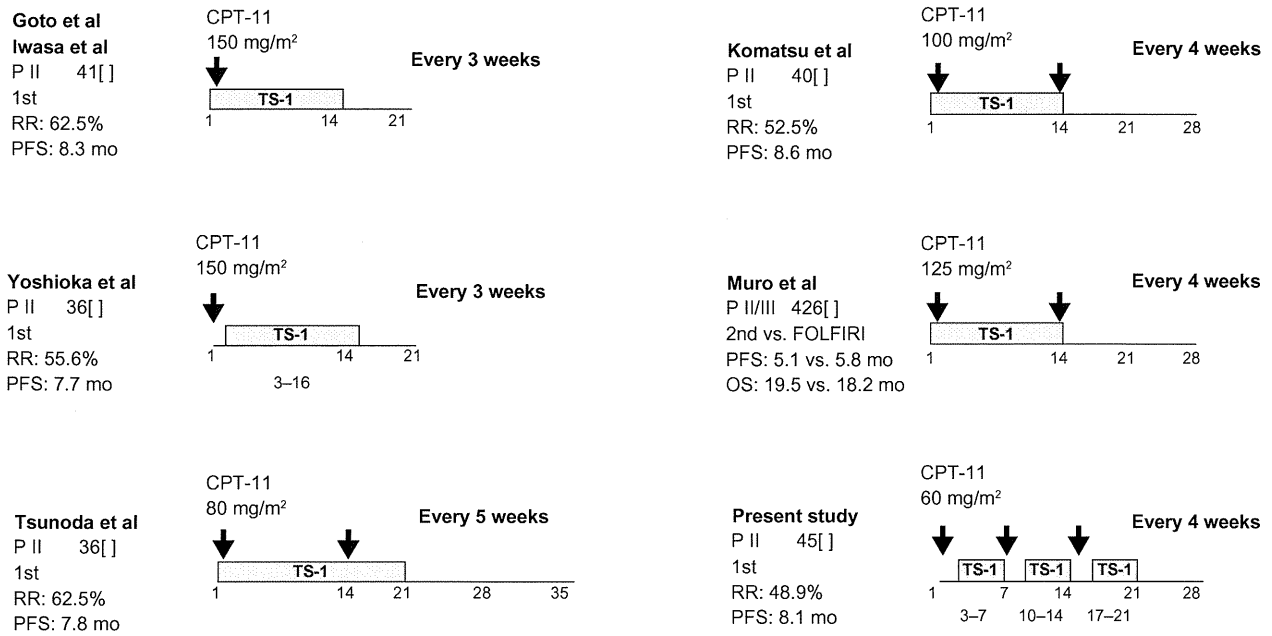

Figure 3. Treatment protocols of various combination chemotherapies of S-1 and irinotecan in advanced or recurrent colorectal cancer.

Since our concept of this new combination schedule consisted of inhibition of tumor angiogenesis and cytotoxic activity, optimal biological doses of cytotoxic agents should be determined using both toxicity and surrogate marker for antiangiogenesis such as CEPs.[31] Moreover, antitumor efficacy of the new schedule may be significantly increased when administered in combination with bevacizumab, an antiangiogenic biologic that is used worldwide for colorectal cancer.[32]

Another advantage of our regimen is the interval of administration of CPT-11 and S-1. The in vitro studies have shown that CPT-11 downregulates thymidylate synthase expression in tumor cells leading to synergy between CPT-11 and 5-FU that was maximal when CPT-11 was given 24 hours prior to 5-FU.[33,34] Therefore, the weekly administration of CPT-11 followed by S-1 with a 2-day interval in our regimen seems to be reasonable in terms of the cytotoxic activity and of gastrointestinal toxicity such as anorexia, nausea, and vomiting. Yoshioka's regimen using the 2-day interval between CPT-11 and S-1 administrations also resulted in a low toxicity.[29]

Toxicity was generally mild and manageable on an outpatient basis. The most common hematological toxicity was neutropenia. However, the incidence of grade 3 or 4 neutropenia was low. The most common types of nonhematological toxicity were diarrhea and anorexia, which were not severe. The incidences of grade 3 or 4 diarrhea and anorexia were also low. Of interest, however, patients with anorexia had many other related adverse effects, such as diarrhea, dehydration, fatigue, and neutropenia (data not shown). In patients who had moderate anorexia or diarrhea, treatment with S-1 was temporarily discontinued. Consequently, grade 2 of either neutropenia or leucopenia was the most common reason for skipping the day 15 dose of CPT-11 in the treatment cycle. However, it was rare that the start of the next treatment cycle was delayed. Neutropenia, diarrhea, nausea, and vomiting frequently occurred in previous studies of combined treatment with CPT-11 plus infusional 5-FU/LV[4,25–28] or with metronomic administration of S-1 and MTD administration of CPT-11.[15–19] Our results suggested that both the incidences and intensities of these toxic reactions with S-1 plus weekly CPT-11 were lower than those with a combination of CPT-11 plus infusional 5-FU/LV or with metronomic administration of S-1 and MTD administration of CPT-11.

The low toxicity in the present study has resulted in higher relative dose intensity. The mean relative dose intensity of both S-1 and CPT-11 exceeded 90% up to 6th cycle. The relative dose intensity of S-1 and CPT-11 in our study was higher than that of combination therapy with metronomic administration of S-1

and MTD administration of CPT-11.[15] In Tsunoda's regimen,[16,35] S-1 was administered twice daily for 3 weeks in combination with MTD administration of CPT-11 on days 1 and 15 of a 5-week cycle. The recommended dose was 80 mg/m^2 of CPT-11. The dose intensity of CPT-11 in a 5-week schedule was very similar to that with Goto's regimen.[15] These findings suggest that the use of higher doses of CPT-11 would probably require a lower dose of S-1 or temporary discontinuation of S-1 to control toxicity, especially neutropenia, diarrhea, or prolonged fatigue, within acceptable levels.

Capecitabine is a widely used oral fluoropyrimidine derivative. Studies of a combination of capecitabine plus CPT-11 have shown significant efficacy, response rates ranging from 47% to 61%, and a median PFS or TTP of 6.1 to 8.3 months in patients with colorectal cancer.[36,37] However, the incidence of grade 3 or 4 diarrhea with capecitabine plus CPT-11 was greater than 20%, clearly higher than that with our study and other regimens with S-1 and CPT-11. Both CPT-11 and capecitabine are metabolized by carboxylesterases in the liver to an active metabolite, 7-ethyl-10-hydroxy-camptothecin (SN-38), and to an intermediate metabolite, 5′-deoxy-5-fluoropyrimidine, respectively. The complex metabolism of both capecitabine and CPT-11 can thus theoretically lead to pharmacokinetic drug-drug interactions.[38] In contrast, a previous phase I trial using S-1 and CPT-11 showed no change in the plasma concentrations of 5-FU, FBAL, or SN-38 as compared with the concentrations after administration of S-1 or CPT-11 alone.[39] When CPT-11 is combined with S-1, it may, therefore, be safer and more convenient than a combination of capecitabine and CPT-11.

Conclusion

New combination chemotherapy using daily S-1 and low-dose weekly CPT-11 appeared to be an effective, well-tolerated, and convenient regimen in patients with advanced colorectal cancer. Our findings suggest that this new combination chemotherapy is a promising regimen, offering benefits in terms of safety and survival as compared with conventional regimens. Future studies must objectively confirm that the new S-1 and CPT-11 combination therapy can replace the standard FOLFIRI without negatively affecting efficacy or safety.

Acknowledgements

We are indebted to the physicians and all other comedical staff who contributed to this study.

Funding

This work was supported by a Grant-in-Aid for Scientific Research (C) (No. 20591597) from the Ministry of Education, Culture, Sports, Science and Technology, of Japan.

Author Contributions

The principal investigator: KS. Responsible for study concept and design: YO. Provided patients: YO, TT, YA, NI, YT, KM, MI, SS, and AK. Collected and analyzed the data: YO, YA, and NI. Interpreted the data: YO, YA, and KS. Wrote the manuscript: YO. All authors reviewed and approved the final manuscript.

Competing Interests

Author(s) disclose no potential conflicts of interest.

Disclosures and Ethics

As a requirement of publication author(s) have provided to the publisher signed confirmation of compliance with legal and ethical obligations including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclosures are made in this section. The external blind peer reviewers report no conflicts of interest.

This phase II study was conducted to validate the antitumor efficacy and safety of a new combination schedule of weekly low-dose irinotecan and daily S-1. The new combination schedule is an effective, less toxic, and convenient regimen in patients with advanced colorectal cancer.

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EUROPEAN JOURNAL OF CANCER 48 (2012) 1407–1413

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Goshajinkigan reduces oxaliplatin-induced peripheral neuropathy without affecting anti-tumour efficacy in rodents

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ARTICLE INFO

Article history:
Available online 8 September 2011

Keywords:
Oxaliplatin
Neuropathy
Allodynia
Hyperalgesia
Goshajinkigan
Anti-tumour activity

ABSTRACT

Oxaliplatin is a key drug in the treatment of colorectal cancer, but it causes acute and chronic neuropathies in patients. Goshajinkigan (GJG) is a Kampo medicine that is used for the treatments of several neurological symptoms including pain and numbness. More recently, GJG has been reported to prevent the oxaliplatin-induced peripheral neuropathy in clinical studies. No experimental study, however, has been conducted to date to determine the effect of GJG on pain behaviour in a rat model of oxaliplatin-induced neuropathy. Moreover, the impact on the anti-tumour effect of oxaliplatin remains unknown. In the present study, we examined the effects of GJG on the peripheral neuropathy and anti-tumour activity of oxaliplatin in rodents. Repeated administration of oxaliplatin caused cold hyperalgesia from days 3 to 37 and mechanical allodynia from days 21 to 28. Repeated administration of GJG prevented the oxaliplatin-induced cold hyperalgesia but not mechanical allodynia and axonal degeneration in rat sciatic nerve. Single administration of GJG reduced both cold hyperalgesia and mechanical allodynia after the development of neuropathy. In addition, GJG did not affect the anti-tumour effect of oxaliplatin in the tumour cells or tumour cells-implanted mice. These results suggest that GJG relieves the oxaliplatin-induced cold hyperalgesia and mechanical allodynia without affecting anti-tumour activity of oxaliplatin, and, therefore, may be useful for the oxaliplatin-induced neuropathy in clinical practice.

1. Introduction

Oxaliplatin, a platinum-based chemotherapeutic agent, is widely used for colorectal cancer. However, it causes severe acute and chronic peripheral neuropathies. Acute neuropathy is peculiar to oxaliplatin and includes acral paresthesias enhanced by exposure to cold.[1–4] The acute neuropathy is thought to be not due to morphological damage of the nerve.[5,6] On the other hand, the chronic neuropathy is characterised by sensory and motor neuropathy after long-term treatment with oxaliplatin and it is similar to cisplatin-induced neurological symptom.[3] This chronic neuropathy is often a dose-limiting toxicity.[7,8] For this reason, peripheral neuropathy associated with the administration of oxaliplatin is a major clinical problem in chemotherapy.

The OPTIMOX (stop and go) approach offers a reasonably good strategy[9] and attempts to prevent oxaliplatin-induced neuropathy, but it has not been successful well. Gamelin et al.[10,11] reported that intravenous administration of calcium gluconate and magnesium sulphate (Ca/Mg) before and after oxaliplatin therapy could alleviate peripheral neurotoxicity, but the injections of these drugs make the chemotherapy

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doi:10.1016/j.ejca.2011.08.009

regimen cumbersome and complicated. Therefore, a preventive agent for neuropathy has not yet been established. Oxaliplatin is metabolised to oxalate and dichloro(1,2-diaminocyclohexane) platinum [Pt(dach)Cl_2].[12] We previously demonstrated that repeated administration of oxaliplatin (4 mg/kg) induced cold hyperalgesia in the early phase and mechanical allodynia in the late phase in rats, and that oxalate is involved in the cold hyperalgesia but not mechanical allodynia.[13] Moreover, we indicated that pre-administration of Ca or Mg prevents the cold hyperalgesia but not mechanical allodynia which is related to Pt(dach)Cl_2.[13]

Goshajinkigan (GJG), a Kampo medicine, has widely been used to treat symptoms like numbness, vibration sensation, cold sensation and limb pain associated with diabetic neuropathy.[14–16] More recently, GJG has been shown to prevent the oxaliplatin-induced peripheral neuropathy in clinical studies.[17,18] No experimental study, however, has been conducted to date to determine the effect of GJG on pain behaviour in an animal model of oxaliplatin-induced neuropathy. Moreover, the impact on the anti-tumour effect of oxaliplatin remains unknown. In the present study, we examined the effects of GJG on the peripheral neuropathy and anti-tumour activity of oxaliplatin in rodents.

2. Materials and methods

2.1. Animals

Six-week-old male Sprague–Dawley rats weighing 200–250 g (Kyudo Co., Saga, Japan) were used for the oxaliplatin-induced peripheral neuropathy model. Six-week-old male BALB/c mice weighing 21–23 g (CLEA Japan, Inc., Tokyo, Japan) were used for the *in vivo* tumour growth model. They were housed in groups of four to five per cage, with lights on from 07:00 to 19:00 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, and we followed International Association for the Study of Pain (IASP) Committee for Research and Ethical Issues guidelines for animal research.[19]

2.2. Drugs

Oxaliplatin (Elplat®) was obtained from Yakult Co., Ltd. (Tokyo, Japan) and was dissolved in 5% glucose solution. GJG (Lot. No. 2090107010) was a generous gift from Tsumura & CO. (Tokyo, Japan). In the oxaliplatin-induced peripheral neuropathy model, oxaliplatin (4 mg/kg) or vehicle (5% glucose solution) was injected intraperitoneally (i.p.) twice a week for 4 weeks (days 1, 2, 8, 9, 15, 16, 22 and 23). Oxaliplatin was administered at a volume of 1 mL/kg of body weight. GJG (0.3 and 1.0 g/kg) was dissolved in distilled water. The doses of these drugs were chosen based on a previous report.[13,20–23] Behavioural tests were performed blindly with respect to drug administration.

2.3. Acetone test for cold hyperalgesia

The cold hyperalgesia was assessed by acetone test described by Flatters and Bennett.[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. 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, Unites States of America), and the number of withdrawal response was counted for 40 s from the start of the acetone spray.

2.4. von Frey test for mechanical allodynia

The mechanical allodynia was assessed by von Frey test. 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, United Kingdom) ranging 1–15 g bending force were applied to the midplantar skin of each hind paw with each application held for 6 s. Withdrawal responses to the stimulation of von Frey filaments were monitored and paw withdrawal thresholds were determined by a modified up-down method.[23]

2.5. Experimental schedule

To examine the preventive effect of repeated administration of GJG on the oxaliplatin-induced cold hyperalgesia and mechanical allodynia, GJG was administered p.o. once a day for 4 weeks. The acetone test was performed before the first drug administration (on day 0) and on days 1, 3, 5, 7, 14, 21, 30 and 37. On days 1, 3, 5, 7, 14 and 21, this test was performed before drug administration. The von Frey test was performed before the first drug administration (on day 0) and on days 5, 15, 21 and 28. This behavioural test was performed before drug administration.

Next, we examined the therapeutic effect of single administration of GJG on the oxaliplatin-induced cold hyperalgesia and mechanical allodynia after the development of neuropathy. We confirmed the incidence of cold hyperalgesia and mechanical allodynia on day 5 and day 28, respectively. We carried out the drug evaluation the next day. GJG was administered p.o. The acetone test was performed immediately before (0 min) and at 30, 60, 90, 120, 150 and 180 min after administration. The von Frey test was performed immediately before (0 min) and at 30, 60, 90 and 120 min after administration. GJG was administered at a volume of 5 mL/kg of body weight.

2.6. Assay of sciatic nerve axonal degeneration

On day 28, sciatic nerves were harvested from rats anesthetised 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 wash 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 (BX51; Olympus Corp., Tokyo, Japan).

2.7. Cell cultures

Murine colon adenocarcinoma 26 (C-26) cells were obtained from the Riken (Saitama, Japan). C-26 cells were maintained

in RPMI 1640 medium (MP Biomedicals Inc., Irvine, CA, USA) containing 2 mM L-glutamine, 10% foetal bovine serum in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.8. *Tumour cytotoxicity assay*

C-26 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 oxaliplatin (10 ng/mL) and GJG (10, 30, 100,or 300 μg/mL) for 12, 24 or 48 h. Oxaliplatin and GJG were dissolved in medium. The cell viability was assessed by the mitochondrial activity in reducing WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2 H-tetrazolium, monosodium salt) to formazan. At 12, 24, or 48 h after incubation with oxaliplatin and GJG, 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).

2.9. *Tumour growth analysis using mouse model*

C-26 cells (1.0×10^6 cells per mouse in 10 μL serum free medium) were implanted subcutaneously in the left paw of

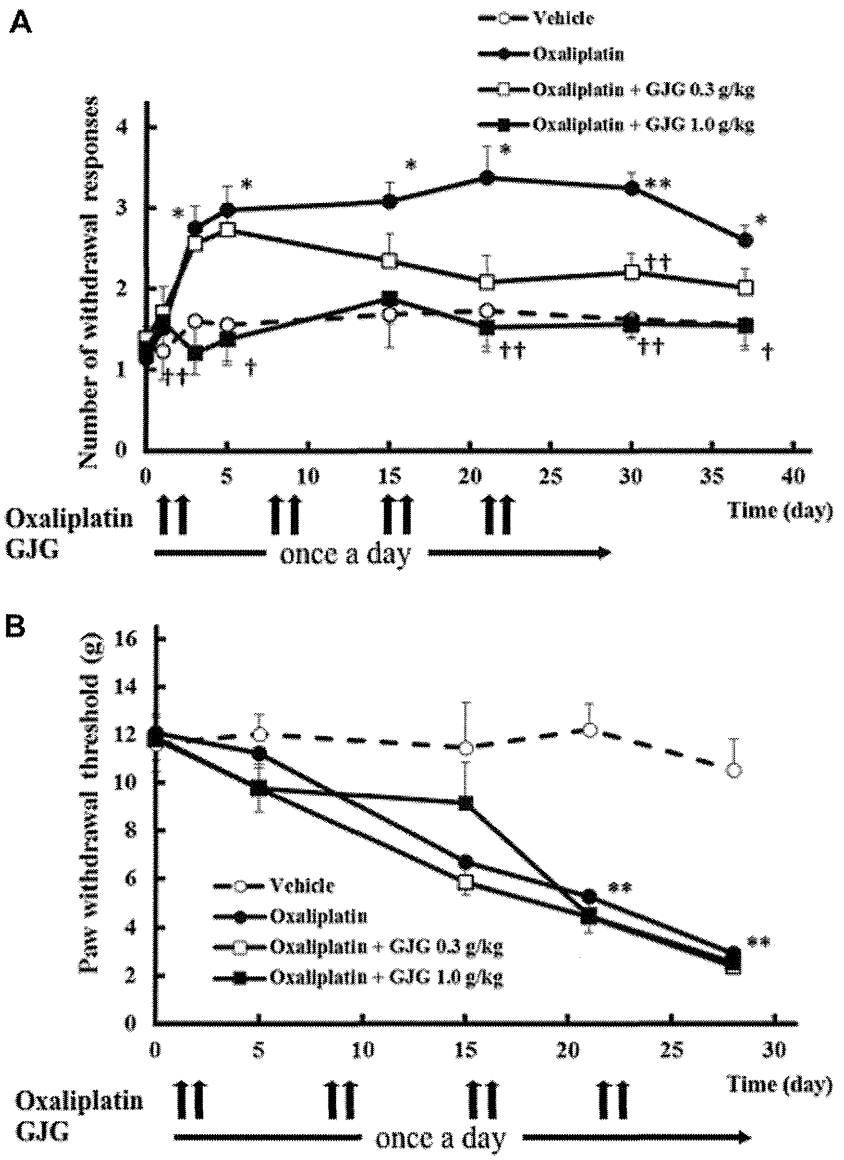

Fig. 1 – Effects of repeated administration of goshajinkigan (GJG) on oxaliplatin-induced cold hyperalgesia and mechanical allodynia in acetone (A) and von Frey (B) tests in rats. Oxaliplatin (4 mg/kg) was administered i.p. twice a week for 4 weeks. GJG (0.3 and 1.0 g/kg) was administered p.o. once a day for 4 weeks. The acetone test was performed before the first drug administration (on day 0) and on days 1, 3, 5, 7, 14, 21, 30 and 37. The von Frey test was performed before the first drug administration (on day 0) and on days 5, 15, 21 and 28. Values are expressed as the mean ± standard error mean of 7–8 animals. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with vehicle. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ compared with oxaliplatin alone.

BALB/c mice. Three days after implantation of tumour cells, administration of drugs was started. Oxaliplatin (6 mg/kg, i.p.) was injected twice a week and GJG (1.5 g/kg, p.o.) was injected once a day. The tumour volumes were calculated as follows: Volume (mm^3) = 1/2 × Thickness (mm) × Length (mm) × Width (mm).

2.10. *Statistical analyses*

Values were expressed as the mean ± standard error mean. The values were analysed by the Student's *t*-test, or 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 percentages of level of vehicle-treated group. A probability level of $P < 0.05$ was accepted as statistically significant.

3. Result

3.1. *Effect of repeated administration of GJG on cold hyperalgesia in acetone test in oxaliplatin-treated rats*

Oxaliplatin (4 mg/kg, i.p., twice a week) significantly increased the number of withdrawal responses compared with vehicle on days 3, 5, 15, 21, 30 and 37 ($P < 0.05$ or 0.01 by Tukey–Kramer test, Fig. 1A). The repeated administration of GJG (0.3 g/kg, p.o.) weakly reduced the increase of number of withdrawal responses by oxaliplatin (day 30: $P < 0.01$ by Tukey–Kramer test). Moreover, GJG (1.0 g/kg, p.o.) completely reversed the oxaliplatin-induced increase of number of withdrawal responses (days 5 and 37: $P < 0.05$, days 3, 21 and 30: $P < 0.01$ by Tukey–Kramer test).

3.2. *Effect of repeated administration of GJG on mechanical allodynia in von Frey test in oxaliplatin-treated rat*

Oxaliplatin (4 mg/kg, i.p., twice a week) significantly reduced the withdrawal threshold compared with vehicle on days 21 and 28 ($P < 0.01$ by Tukey–Kramer test, Fig. 1B). The repeated administration of GJG (0.3 and 1.0 g/kg) had no effect on the oxaliplatin-induced reduction of withdrawal threshold.

3.3. *Effect of repeated administration of GJG on oxaliplatin-induced histological change in rat sciatic nerve*

No histological abnormalities in sciatic nerve were observed in vehicle-treated rats (Fig. 2). Oxaliplatin (4 mg/kg, i.p., twice a week) induced the decrease in the density of myelinated fibres and the degeneration of myelinated fibres in rat sciatic nerve. These histological changes were also observed in the tissue of rat treated with co-administration of oxaliplatin and GJG.

3.4. *Effect of single administration of GJG on cold hyperalgesia after the development of neuropathy in acetone test in oxaliplatin-treated rats*

Oxaliplatin (4 mg/kg, i.p., twice on days 1 and 2) significantly increased the number of withdrawal responses compared with vehicle in acetone test on day 5 ($P < 0.05$ by Tukey–Kramer test, Fig. 3A). The single administration of GJG (0.3 g/kg) had no effect on the oxaliplatin-induced increase of number of withdrawal responses, while GJG (1.0 g/kg) significantly reduced this response (30 and 90 min: $P < 0.05$, 60 min: $P < 0.01$ by Tukey–Kramer test). This effect of GJG disappeared by 180 min after administration.

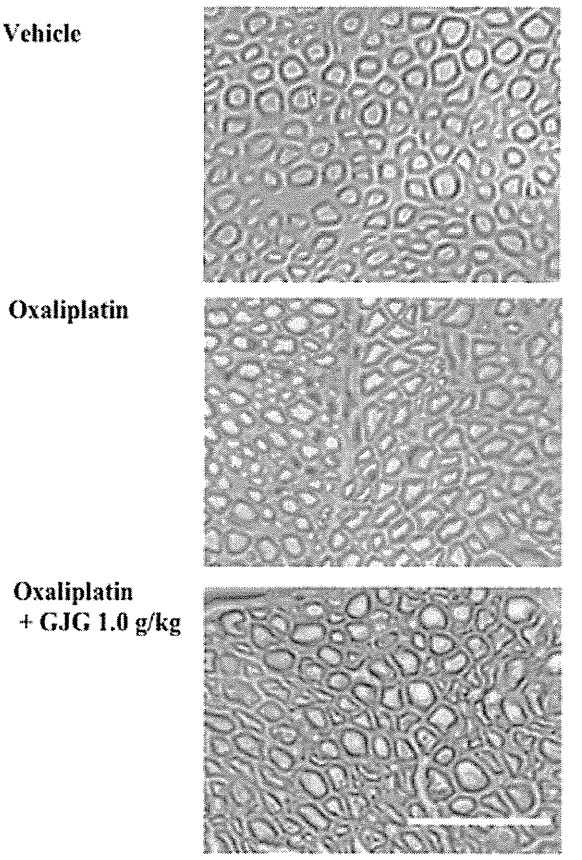

Fig. 2 – Effect of repeated administration of goshajinkigan (GJG) on histological change induced by oxaliplatin in rat sciatic nerve. Rats were treated with oxaliplatin (4 mg/kg, i.p.) twice a week for 4 weeks. GJG (1.0 g/kg) was administered p.o. once a day for 4 weeks. On day 28, the sciatic nerve was harvested, and samples were stained with toluidine blue. Photographs were originally magnified 800×. Scale bar 50 μm.

3.5. *Effect of single administration of GJG on mechanical allodynia after the development of neuropathy in von Frey test in oxaliplatin-treated rats*

Oxaliplatin (4 mg/kg, i.p., twice on days 1, 2, 8, 9, 15, 16, 22 and 23) significantly reduced the withdrawal threshold compared with vehicle on day 28 ($P < 0.01$ by Tukey–Kramer test, Fig. 3B). The single administration of GJG (0.3 g/kg) significantly increased the reduced threshold by oxaliplatin at 30 and 90 min after administration ($P < 0.05$ by Tukey–Kramer test). Similarly, GJG (1.0 g/kg) significantly increased the oxaliplatin-induced reduction of withdrawal threshold at 30 min after administration ($P < 0.01$ by Tukey–Kramer test). These effects of GJG disappeared by 120 min after administration.

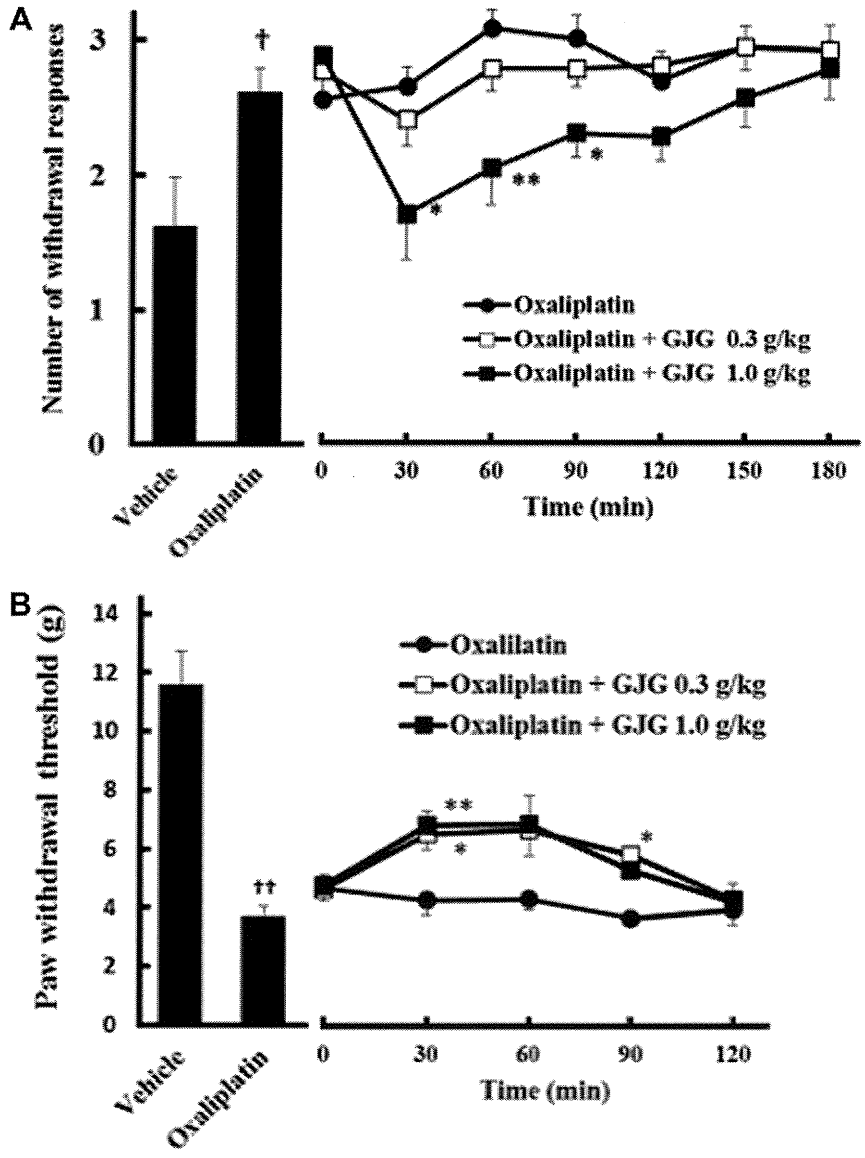

Fig. 3 – Effects of single administration of goshajinkigan (GJG) on the cold hyperalgesia and mechanical allodynia after the development of neuropathy in acetone (A) and von Frey (B) tests in oxaliplatin-treated rats. Rats were treated with oxaliplatin (4 mg/kg, i.p.) twice on days 1 and 2 (A) or twice on days 1, 2, 8, 9, 15, 16, 22 and 23 (B). We confirmed the incidence of cold hyperalgesia and mechanical allodynia on days 5 and 28, respectively. We carried out the drug evaluation the next day. GJG (0.3 and 1.0 g/kg) was administered p.o. Values are expressed as the mean ± standard error mean of 6–8 animals. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ compared with vehicle. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with oxaliplatin alone.

3.6. *Effect of GJG on the tumour cytotoxicity of oxaliplatin*

The exposure of cultured C-26 cells to oxaliplatin (3 μM) for 12, 24 or 48 h caused time-dependent decreases in tumour cell viability as assessed by mitochondrial enzyme activity using the WST-8 assay (Fig. 4). GJG (10–300 μg/mL) had no effect on the oxaliplatin-induced decrease of tumour cell viability in cell line.

3.7. *Effect of GJG on the anti-tumour activity of oxaliplatin in tumour cells-implanted mice*

Oxaliplatin (6 mg/kg, i.p.) significantly inhibited the increase of tumour volumes compared with vehicle on days 11 and 16 in tumour cells-implanted mice ($P < 0.01$ by Tukey–Kramer test, Fig. 5). GJG (1.5 g/kg, p.o.) had no effect on the oxaliplatin-induced inhibition of tumour growth.

4. Discussion

In the present study, oxaliplatin caused cold hyperalgesia from the early phase and mechanical allodynia in the late phase, consistently with our previous reports.[13,23] The repeated administration of GJG reduced the oxaliplatin-induced cold hyperalgesia in the acetone test, whereas it had no effect on the oxaliplatin-induced mechanical allodynia in the von Frey test. Recently, an increased expression of transient receptor potential melastatin 8 (TRPM8) has been

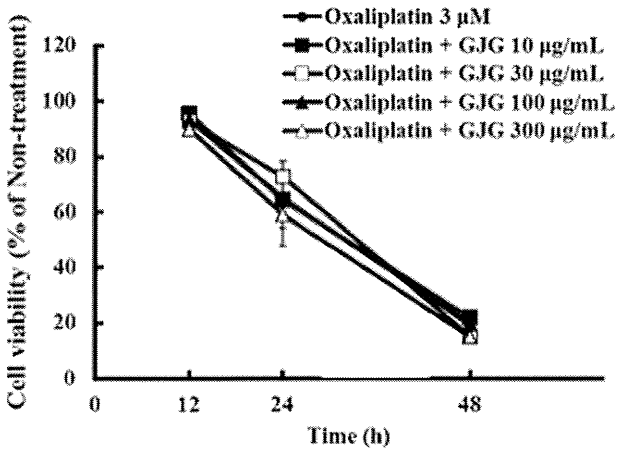

Fig. 4 – Effect of goshajinkigan (GJG) on the tumour cytotoxicity of oxaliplatin. C-26 cells were incubated with oxaliplatin (3 μM) for 12, 24, or 48 h in the presence or absence of various concentrations (10–300 μg/mL) of GJG. Cell viability was measured by WST-8 assay. Values are expressed as percentages of the viability of the vehicle-treated group (n = 6–9).

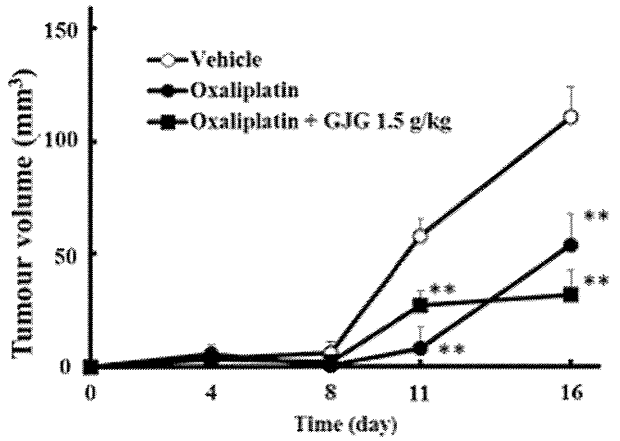

Fig. 5 – Effect of goshajinkigan (GJG) on the anti-tumour effect of oxaliplatin. C-26 cells-implanted mice were treated with oxaliplatin (6 mg/kg, i.p.) twice a week and GJG (1.5 g/kg, p.o.) once a day for 16 days. Values are expressed as the mean ± standard error mean of 12 animals on days 0, 4, 8, 11 and 16. $^{**}P < 0.01$ compared with vehicle.

reported to be involved in oxaliplatin-induced cold allodynia in mice.[25] Single administration of oxaliplatin increases the expression level of TRPM8 mRNA at day 3 after injection and the expression is decreased to the normal level on day 10. The TRPM8 is activated by cooling temperature, and its mRNA is expressed in dorsal root ganglion, but not in other tissues.[26] Therefore, GJG might prevent the oxaliplatin-induced cold hyperalgesia by inhibiting the expression of TRPM8. We also observed that oxaliplatin caused the degeneration and the decrease in the density of myelinated fibres in rat sciatic nerve on day 28. However, repeated administration of GJG had no effect on the histological changes induced by oxaliplatin. These results suggest that GJG cannot protect against the oxaliplatin-induced axonal degeneration. Recently, we have reported that no histological abnormalities in sciatic nerve were observed in oxaliplatin-treated rats on day 5, although oxaliplatin caused cold hyperalgesia in the acetone test on that day.[23] Therefore, it is unlikely that repeated administration of GJG prevented the oxaliplatin-induced cold hyperalgesia by protecting against the axonal degeneration. In addition, the present results support the involvement of axonal degeneration in the incidence of mechanical allodynia in the late phase but not cold hyperalgesia from the early phase.

Our data in this study revealed that single administration of GJG after the development of neuropathy reduced both cold hyperalgesia and mechanical allodynia. The present results suggest that GJG is useful as symptomatic therapy for oxaliplatin-induced peripheral neuropathy. GJG has been reported to show anti-nociceptive effect based on not only stimulation of spinal κ-opioid receptors via dynorphin release but also increase of peripheral blood flow via increase in nitric oxide production, in streptozotocin-induced diabetic mice.[20,21,27] The herbal medicine component of GJG also has antioxidant properties.[28,29] Furthermore, GJG partially reverses C fibre activation through the reduction of the tachykinins, transient receptor potential vanilloid type 1 (TRPV1) channels and P2X3 purine receptors.[30] Therefore, the effects of GJG on the oxaliplatin-induced cold hyperalgesia and mechanical allodynia might be due to increase of peripheral blood flow, stimulate spinal κ-opioid receptors, inhibit oxidative stress or suppress the C fibre activation. In fact, it has been reported that oxaliplatin gradually decreases peripheral blood flow in mice[31] and increases responses of C-fibre nociceptors to mechanical stimulation in rats.[32] Moreover, both systemic and local administration of antioxidants (acetyl-L-carnitine, alpha-lipoic acid or vitamin C) markedly inhibit the oxaliplatin-induced neuropathy.[32]

In this study, repeated administration of GJG (0.3 g/kg) reduced the cold hyperalgesia in the late phase but not early phase. Though the reason for the effect of GJG is unknown, repeated administration of lower dose of GJG might reduce cold hyperalgesia in the late phase through progressive increase of peripheral blood flow without protecting against the axonal degeneration or inhibiting the expression of TRPM8.

The present results also show that GJG had no effect on the oxaliplatin-induced tumour cytotoxicity in C-26 cells. Furthermore, GJG had no effect on the anti-tumour effect of oxaliplatin in tumour cells-implanted mice. Therefore, it is unlikely that GJG influences the anti-tumour effect of oxaliplatin.

In conclusion, the study presented here demonstrates, for the first time, that GJG ameliorates the oxaliplatin-induced neuropathy in the rat model without affecting the anti-tumour activity of oxaliplatin. However, GJG cannot protect against the oxaliplatin-induced axonal degeneration in rat sciatic nerve. Therefore, GJG is expected to be useful as symptomatic therapy for clinical oxaliplatin-induced neuropathy if it is used with particular care of sensory and motor neuropathies. These data are important information for clinical trials of GJG now underway in particular.

Conflict of interest statement

None declared.

Acknowledgements

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). The authors are grateful to Tsumura & CO. (Tokyo, Japan) for generously supplying the GJG.

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J Pharmacol Sci **118**, 547 – 551 (2012)

Journal of Pharmacological Sciences

Short Communication

Repeated Administration of Amitriptyline Reduces Oxaliplatin-Induced Mechanical Allodynia in Rats

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Received January 6, 2012; Accepted February 17, 2012

Abstract. Oxaliplatin is a key drug in the treatment of colorectal cancer, but it causes acute and chronic neuropathies in patients. Amitriptyline has widely been used in patients with painful neuropathy. In this study, we investigated the effect of amitriptyline on the oxaliplatin-induced neuropathy in rats. Repeated administration of amitriptyline (5 and 10 mg/kg, p.o., once a day) reduced the oxaliplatin-induced mechanical allodynia but not cold hyperalgesia and reversed the oxaliplatin-induced increase in the expression of NR2B protein and mRNA in rat spinal cord. These results suggest that amitriptyline is useful for the treatment of oxaliplatin-induced neuropathy clinically.

Keywords: oxaliplatin, amitriptyline, peripheral neuropathy

Oxaliplatin has widely been used as a key drug in the treatment of colorectal cancer. However, it causes severe acute and chronic peripheral neuropathies. Acute neuropathy includes acral paresthesias and dysesthesia triggered or enhanced by exposure to cold, and it appears soon after administration (1). After multiple cycles of treatment, the patients develop chronic neuropathy characterized by sensory and motor dysfunction. This chronic neuropathy is a dose-limiting toxicity and a major clinical problem in oxaliplatin chemotherapy (2).

Antidepressant drugs have been recommended to be used as first-line drugs for the treatment of neuropathic pain (3, 4), and in particular, amitriptyline is demonstrated to possess an analgesic activity for neuropathic pain in many randomized controlled trials (5).

Recently, we reported that repeated administration of oxaliplatin induced cold hyperalgesia in the early phase and mechanical allodynia in the late phase in rats (6), and spinal NR2B-containing *N*-methyl-D-aspartate (NMDA) receptors are involved in the oxaliplatin-induced mechanical allodynia (7). However, the effect of amitriptyline on the oxaliplatin-induced neuropathy remains unexplored. Accordingly, we investigated the effect of amitriptyline on the oxaliplatin-induced cold hyperalgesia and mechanical allodynia in rats. Moreover, we examined the effect of amitriptyline on the oxaliplatin-induced increase in the expression of NR2B protein and mRNA in the rat spinal cord.

Male Sprague-Dawley rats (Kyudo Co., Tosu) were used. Rats were housed in groups of four to five per cage, with lights on from 7:00 AM to 7:00 PM. 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 (Fukuoka) according to the National Institutes of Health guidelines, and we followed the International Association for the Study of Pain (IASP) Committee for Research and Ethical Issues guidelines for animal research (8).

Oxaliplatin (Elplat®) was obtained from Yakult Co., Ltd. (Tokyo) and was dissolved in 5% glucose solution. Amitriptyline was provided by Wako Pure Chemical Industries, Ltd. (Osaka) and was dissolved in distilled water. Oxaliplatin (4 mg/kg) or vehicle (5% glucose solution) was injected intraperitoneally (i.p.) in volumes of 1 mL/kg, twice a week for 4 weeks (days 1, 2, 8, 9, 15, 16, 22, and 23). Amitriptyline (5 and 10 mg/kg) was administered p.o. once a day for 27 days (from day 1). The doses of these drugs were chosen based on previous reports (6, 7, 9). Behavioral tests were performed blindly with respect to drug administration.

The cold hyperalgesia was assessed by acetone test described by Flatters and Bennett (10). The acetone test was performed before the first drug administration (on day 0) and on days 4, 7, 11, 14, 18, 21, and 28. On days

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Published online in J-STAGE on March 29, 2012 (in advance)
doi: 10.1254/jphs.12006SC

4, 7, 11, 14, 18, and 21, test was performed before drug administration. 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 microliters of acetone (Wako Pure Chemical Industries, Ltd.) 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.

The mechanical allodynia was assessed by the von Frey test. The von Frey test was performed before the first drug administration (on day 0) and on days 7, 14, 21, 28, 35, 42, 49, and 56. On days 7, 14, and 21, test was performed before drug administration. 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 with each application held for 6 s. The paw withdrawal threshold was determined by a modified up-down method (11).

To investigate the functional changes in mRNA levels of NR2B, the L4-6 spinal cord was quickly removed on day 28. mRNA was isolated using PolyATtract® System 1000 (Promega, Corp., Madison, WI, USA). cDNA was synthesized using the PrimScript® 1st strand cDNA Synthesis Kit (Takara Bio, Inc., Otsu). The polymerase chain reaction (PCR) was carried out with Gene Taq (Nippon Gene, Co., Ltd., Tokyo). The oligonucleotide primers for NR2B were designed based on the sequences described by Lau et al. (12). The sequences of PCR primers were as follows: NR2B, 5′-TCC GTC TTT CTT ATG GG ATA TGC-3′ (sense), 5′-CCT CTA GGC GGA CAG ATT AAG G-3′ (antisense); glyceraldehyde-3-phosphate dehydrogenase (G3PDH), 5′-YGC CTG CTT CAC CAC CTT-3′ (sense), 5′-TGC MTC CTG CAC CAC CAA CT-3′ (antisense) (Sigma-Aldrich, St. Louis, MO, USA). Reactions were run for 40 cycles with 95°C denaturing cycle (30 s), 63°C annealing cycle (1 min), and 72°C extension cycle (30 s) for NR2B or for 30 cycles with 94°C denaturing cycle (45 s), 53°C annealing cycle (45 s), and 72°C extension cycle (1.5 min) for G3PDH. The PCR products were subjected to electrophoresis on 2% agarose gel, and the DNA was visualized by staining with ethidium bromide under ultraviolet irradiation. Then, the intensities of PCR products were semi-quantified densitometrically by Alpha Imager 2200 (Protein Simple, Santa Clara, CA, USA).

To examine the functional changes in protein levels of NR2B, the L4-6 spinal cord was quickly removed on day 28. The tissues were homogenized in a solubilization buffer containing 20 mM Tris-HCl, 2 mM EDTA, 0.5 mM EGTA, 10 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 0.32 M sucrose, 2 mg/ml aprotinine, and 2 mg/ml leupeptin, pH 7.4. The homogenates were subjected to 6% SDS-PAGE, and proteins were transferred electrophoretically to PVDF membranes. The membranes were blocked in Tris-buffered saline / Tween-20 (TBST) containing 5% BSA (Sigma-Aldrich) for an additional 1 h at room temperature with agitation. The membrane was incubated overnight at 4°C with rabbit polyclonal NR2B antibody (1:5000; Millipore, Corp., Billerica, MA, USA) and then incubated for 1 h with anti-rabbit IgG–horseradish peroxidase (1:5000; Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA). The immunoreactivity was detected using Enhanced Chemiluminescence (Perkin Elmer, Waltham, MA, USA).

Values were expressed as the mean ± S.E.M. The values were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer post hoc test to determine differences among the groups. A probability level of $P < 0.05$ was accepted as statistically significant.

In the acetone test, oxaliplatin (4 mg/kg, i.p., twice a week) significantly increased the number of withdrawal responses compared with vehicle on days 11, 14, 18, 21, and 28 (days 11, 14, and 18: $P < 0.05$, days 21 and 28: $P < 0.01$; Fig. 1A). Repeated administration of amitriptyline (5 and 10 mg/kg, p.o., once a day) did not affect the oxaliplatin-induced increase in the number of withdrawal responses.

In the von Frey test, oxaliplatin significantly reduced the withdrawal threshold compared with vehicle on days 14, 21, 28, 35, 42, 49, and 56 ($P < 0.01$, Fig. 1B). Repeated administration of amitriptyline (10 mg/kg) significantly inhibited the oxaliplatin-induced reduction of the withdrawal threshold on days 14, 21, 28, 35, 42, 49, and 56 ($P < 0.01$). In addition, repeated administration of amitriptyline (5 mg/kg) significantly inhibited the oxaliplatin-induced reduction of the withdrawal threshold (day 28: $P < 0.05$ and days 35 and 42: $P < 0.01$).

NR2B expression was examined by western blot and PCR analysis on homogenates of the spinal cord from rats. The results of western blotting and PCR showed that NR2B protein and mRNA levels in the spinal cord of oxaliplatin-treated rats significantly increased compared with that of vehicle-treated rats on day 28 ($P < 0.05$, Fig. 2). Repeated administration of amitriptyline (5 and 10 mg/kg) significantly reversed the oxaliplatin-induced increase in the NR2B mRNA levels in the spinal cord ($P < 0.05$, Fig. 2A). Similarly, repeated administration of amitriptyline (10 mg/kg) significantly reversed the oxaliplatin-induced increase in the NR2B protein levels in the spinal cord ($P < 0.01$, Fig. 2B). In addition, repeated

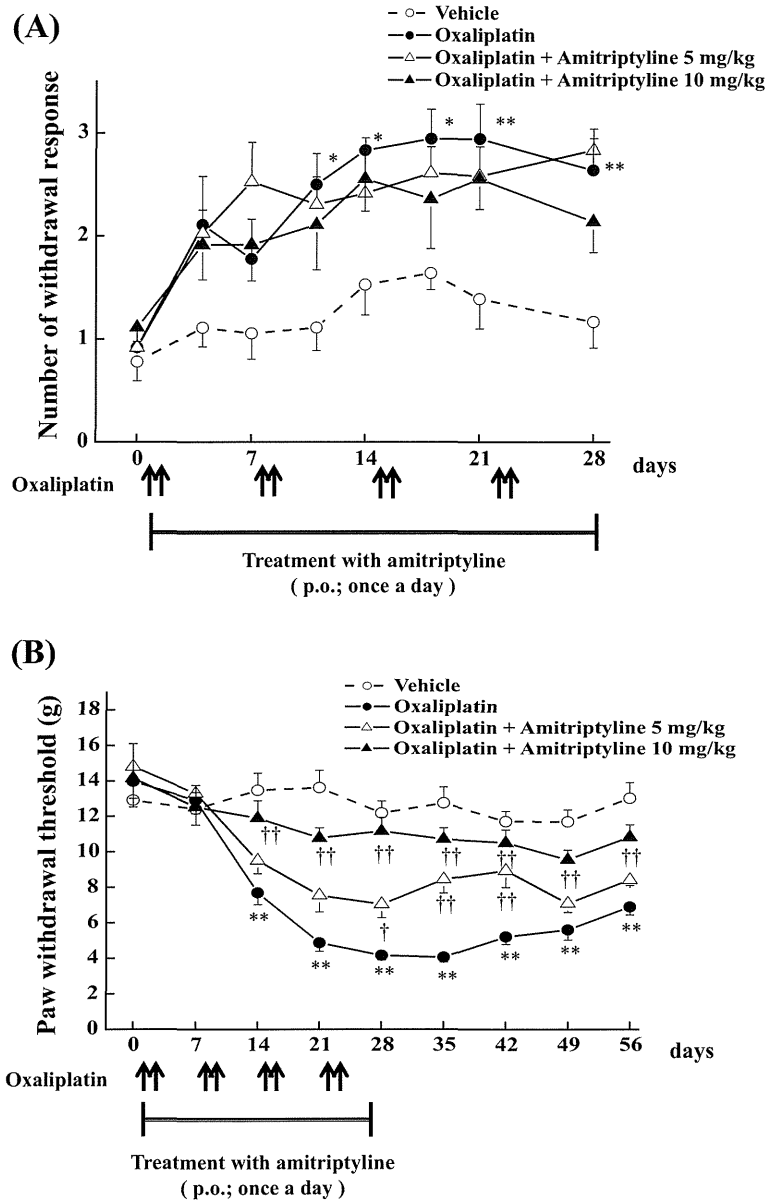

Fig. 1. Effects of amitriptyline on oxaliplatin-induced cold hyperalgesia in the acetone test (A) and mechanical allodynia in the von Frey test (B) in rats. Oxaliplatin (4 mg/kg) was administered i.p. twice a week for 4 weeks (days 1, 2, 8, 9, 15, 16, 22, and 23). Amitriptyline (5 and 10 mg/kg) was administered p.o. once a day for 27 days. The acetone test was performed before the first drug administration (on day 0) and on days 4, 7, 11, 14, 18, 21, and 28. The von Frey test was performed before the first drug administration (on day 0) and on days 7, 14, 21, 28, 35, 42, 49, and 56. Values are expressed as the mean ± S.E.M. of 6 animals (A) or 9 – 10 animals (B). $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the vehicle; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, compared with oxaliplatin alone.

administration of amitriptyline (5 mg/kg) tended to reduce the oxaliplatin-induced increase in the NR2B protein levels.

In this study, repeated administration of amitriptyline reduced the oxaliplatin-induced mechanical allodynia but not cold hyperalgesia. Therefore, amitriptyline may be useful for treatment of the oxaliplatin-induced chronic peripheral neuropathy.

Furthermore, repeated administration of amitriptyline reversed the oxaliplatin-induced increase in the NR2B protein and mRNA levels in the spinal cord. Tricyclic antidepressant agents (TCAs) have been reported to have properties to bind NMDA receptors and to inhibit binding of NMDA ligand (13, 14). Amitriptyline has also been reported to inhibit NMDA-induced pain behavior in rats (15). Recently, we reported that spinal NR2B-containing NMDA receptors are involved in the oxaliplatin-induced mechanical allodynia (7). Taken together, the reduction of amitriptyline on the expression of NR2B subunits may be involved in its inhibitory effect on the development of oxaliplatin-induced mechanical allodynia.

In this study, we did not evaluate the acute effect of amitriptyline on pain behaviors. Furthermore, its inhibitory effect on the development of oxaliplatin-induced mechanical allodynia persisted up to 56 days after the end of amitriptyline administration. These results suggest that the preventive effect of amitriptyline is not a tran-

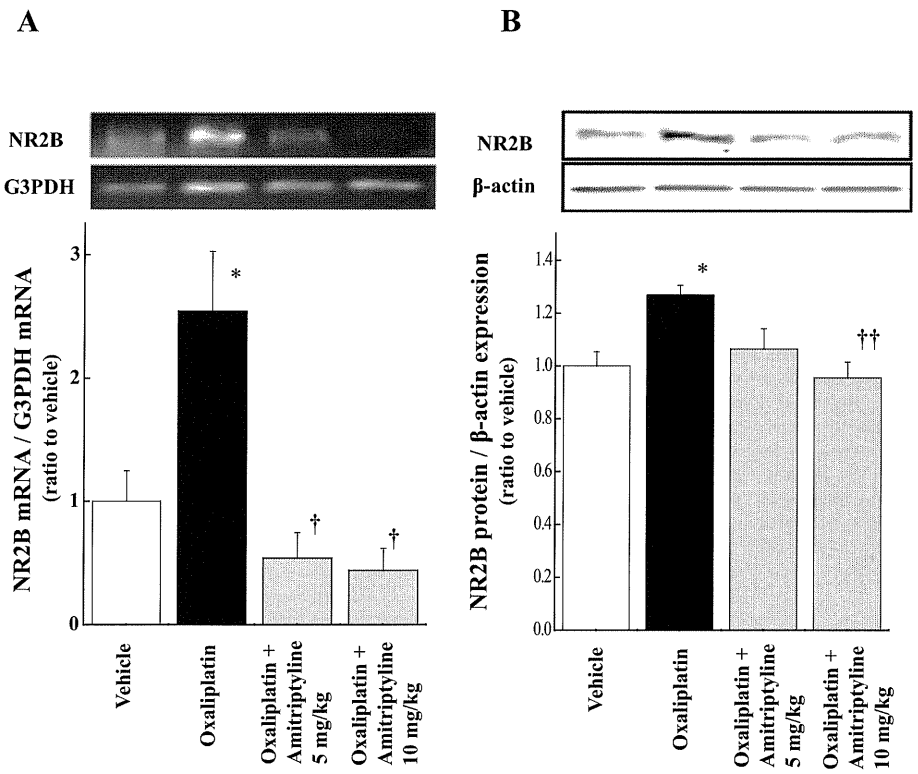

Fig. 2. Effects of amitriptyline on NR2B mRNA (A) and protein (B) levels in the spinal cord in oxaliplatin-treated rats. Oxaliplatin (4 mg/kg) was administered i.p. twice a week for 4 weeks (days 1, 2, 8, 9, 15, 16, 22, and 23). Amitriptyline (5 and 10 mg/kg) was administered p.o. once a day for 27 days. The L4-L6 spinal cord was harvested on day 28. Values are expressed as the mean ± S.E.M. of 5 – 7 animals (A) or 9 – 10 animals (B). $^{*}P < 0.05$, compared with the vehicle; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, compared with oxaliplatin alone.

sient analgesic effect via activation of the descending pain inhibitory system related to monoamine reuptake inhibition and likely due to inhibition of the expression of NR2B subunits. In addition, chronic administration of imipramine for 16 days has been reported to reduce the expression of NMDA-receptor subunit mRNA in mouse brain (16). Therefore, imipramine has the potential to prevent the oxaliplatin-induced mechanical allodynia.

In the present study, we observed that the oxaliplatin-induced mechanical allodynia was gradually recovered on days 42 – 56 after the end of oxaliplatin administration. The present result is consistent with the previous one (11). Clinically, the reversibility of sensory neurotoxicity is observed in patients treated with oxaliplatin (17).

In conclusion, the present results suggest, for the first time, that repeated administration of amitriptyline reduces the oxaliplatin-induced mechanical allodynia, at least in part, by inhibiting the expression of NR2B subunits.

Acknowledgments

Part of this study was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (Nos. 21590285 and 22590242). We thank the Research Support Center, Graduate School of Medical Sciences, Kyushu University for technical support.

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Shirahama *et al. Molecular Pain* 2012, **8**:26
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MOLECULAR PAIN

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Inhibition of Ca^{2+}/Calmodulin-dependent protein kinase II reverses oxaliplatin-induced mechanical allodynia in Rats

Masafumi Shirahama, Soichiro Ushio, Nobuaki Egashira*, Shota Yamamoto, Hikaru Sada, Ken Masuguchi, Takehiro Kawashiri and Ryozo Oishi

Abstract

Background: Oxaliplatin is a key drug in the treatment of colorectal cancer, but it causes severe peripheral neuropathy. We previously reported that oxaliplatin (4 mg/kg, i.p., twice a week) induces mechanical allodynia in the late phase in rats, and that spinal NR2B-containig *N*-methyl-$_D$-aspartate (NMDA) receptors are involved in the oxaliplatin-induced mechanical allodynia. In the present study, we investigated the involvement of Ca^{2+}/calmodulin dependent protein kinase II (CaMKII), which is a major intracellular protein kinase and is activated by NMDA receptor-mediated Ca^{2+} influx, in the oxaliplatin-induced mechanical allodynia in rats.

Results: An increase of CaMKII phosphorylation was found in the spinal cord (L_{4-6}) of oxaliplatin-treated rats. This increased CaMKII phosphorylation was reversed by intrathecal injection of a selective CaMKII inhibitor KN-93 (50 nmol, i.t.) and a selective NR2B antagonist Ro 25-6981 (300 nmol, i.t.). Moreover, acute administration of KN-93 (50 nmol, i.t.) strongly reversed the oxaliplatin-induced mechanical allodynia in von Frey test, while it did not affect the oxaliplatin-induced cold hyperalgesia in acetone test. Similarly, oral administration of trifluoperazine (0.1 and 0.3 mg/kg, p.o.), which is an antipsychotic drug and inhibits calmodulin, reduced both mechanical allodynia and increased CaMKII phosphorylation. On the other hand, trifluoperazine at the effective dose (0.3 mg/kg) had no effect on the paw withdrawal threshold in intact rats. In addition, trifluoperazine at the same dose did not affect the motor coordination in rota-rod test in intact and oxaliplatin-treated rats.

Conclusions: These results suggest that CaMKII is involved in the oxaliplatin-induced mechanical allodynia, and trifluoperazine may be useful for the treatment of oxaliplatin-induced peripheral neuropathy in clinical setting.

Background

Oxaliplatin, a platinum-based chemotherapeutic agent, has widely been used for colorectal cancer. However, oxaliplatin causes severe peripheral neuropathy. After multiple cycles, the patients develop a chronic neuropathy that is characterized by a sensory and motor dysfunction. This chronic neuropathy is a dose-limiting toxicity and a major clinical problem in oxaliplatin-based chemotherapy [1].

We previously reported that repeated administration of oxaliplatin induced cold hyperalgesia in the early phase and mechanical allodynia in the late phase in rats [2]. Recently, we reported that spinal NR2B-containing *N*-methyl-$_D$-aspartate (NMDA) receptors are involved in the oxaliplatin-induced mechanical allodynia [3]. The NMDA receptor antagonists (MK-801 and memantine) and selective NR2B antagonists (Ro25-6981 and ifenprodil) reverse the oxaliplatin-induced mechanical allodynia. In addition, an expression of NR2B protein and mRNA in the rat spinal cord is increased by oxaliplatin on day 25 (late phase).

Activation of the NMDA receptors leads to an increase in Ca^{2+} influx into the cytosol. This increased Ca^{2+} influx initiates cascades of intracellular signaling events involving Ca^{2+} and various protein kinases [4]. Ca^{2+}/calmodulin dependent protein kinase II (CaMKII)

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is a major intracellular protein kinase and is activated by Ca^{2+} signaling [5]. An increase in intracellular Ca^{2+} initially activates calmodulin by binding to its Ca^{2+}-binding sites, and this interaction induces a change in the conformation of calmodulin. CaMKII is then switched to an activated state by exposure to Ca^{2+}/calmodulin. Several studies showed that an increase of CaMKII activation in the spinal cord is involved in persistent pain by nerve injury [6-9] and inflammation [10,11]. However, the role of CaMKII in the oxaliplatin-induced mechanical allodynia still remains unclear. In this study, we investigated the involvement of CaMKII

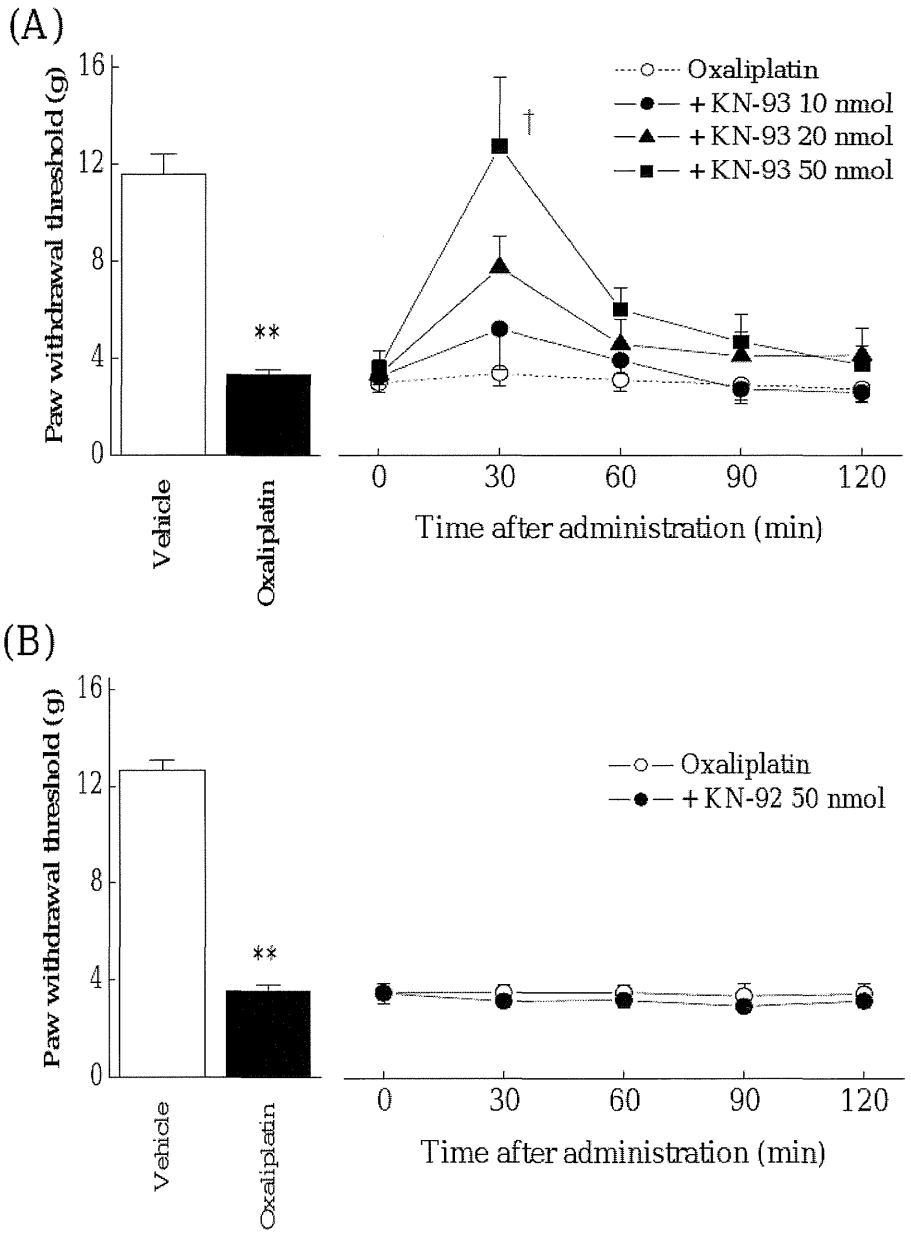

Figure 1 Effects of KN-93 and KN-92 on oxaliplatin-induced mechanical allodynia in the von Frey test. Rats were treated with oxaliplatin (4 mg/kg, i.p.) twice a week for 4 weeks (days 1, 2, 8, 9, 15, 16, 22 and 23). We confirmed the incidence of mechanical allodynia on day 24. We carried out the drug evaluation on the next day. KN-93 (10-50 nmol) or KN-92 (50 nmol) was administered intrathecally. The von Frey test was performed immediately before (0 min) and at 30, 60, 90 and 120 min after administration. KN-93 (50 nmol) significantly reversed oxaliplatin-induced mechanical allodynia (A). On the other hand, KN-92 (50 nmol) had no effect on the mechanical allodynia (B).Values are expressed as the mean ± SEM. of 5-8 animals. $^{**}p < 0.01$ compared with vehicle (Student's *t*-test). †$p < 0.05$ compared with oxaliplatin alone (one-way ANOVA followed by Tukey-Kramer post-hoc test).

in the oxaliplatin-induced mechanical allodynia, and explored novel useful therapeutic drugs for the oxaliplatin-induced neuropathy.

Results

Effects of KN-93 and KN-92 on Oxaliplatin-induced mechanical allodynia

Oxaliplatin (4 mg/kg, i.p., twice a week for 4 weeks) significantly reduced the paw withdrawal thresholds compared with the vehicle in the von Frey test on day 24 ($p < 0.01$, Figure 1). Before administration of KN-93, each group had equivalent paw withdrawal thresholds. The selective CaMKII inhibitor KN-93 (50 nmol, i.t.) completely reversed the reduction of paw withdrawal thresholds by oxaliplatin at 30 min after the administration ($p < 0.05$, Figure 1A). This effect of KN-93 was disappeared within 120 min after the administration. On the other hand, treatment of KN-92 (50 nmol, i.t.), the negative control of KN-93, had no effect on the oxaliplatin-induced mechanical allodynia (Figure 1B).

Effect of KN-93 on Oxaliplatin-induced cold hyperalgesia

Oxaliplatin (4 mg/kg, i.p. on days 1 and 2) significantly increased the number of withdrawal responses to cold stimulation by acetone spray on day 5 ($p < 0.01$, Figure 2). Before administration of KN-93, each group had equivalent number of withdrawal responses. KN-93 (50 nmol, i.t.) did not affect the increase in withdrawal responses by oxaliplatin.

Effects of KN-93 and Ro 25-6981 on Oxaliplatin-induced increase in spinal CaMKII phosphorylation

To determine the effect of oxaliplatin on spinal CaMKII activity, we investigated the expression of CaMKII phosphorylation (pCaMKII). The pCaMKII in the spinal cord of oxaliplatin treated rats significantly increased compared with that of vehicle-treated rats on day 25 ($p < 0.05$, Figure 3). This increased pCaMKII was blocked by the selective CaMKII inhibitor KN-93 (50 nmol, i.t.) and the selective NR2B antagonist Ro 25-6981 (300 nmol, i.t.). (KN-93: $p < 0.05$; Ro 25-6981: $p < 0.01$, Figure 3).

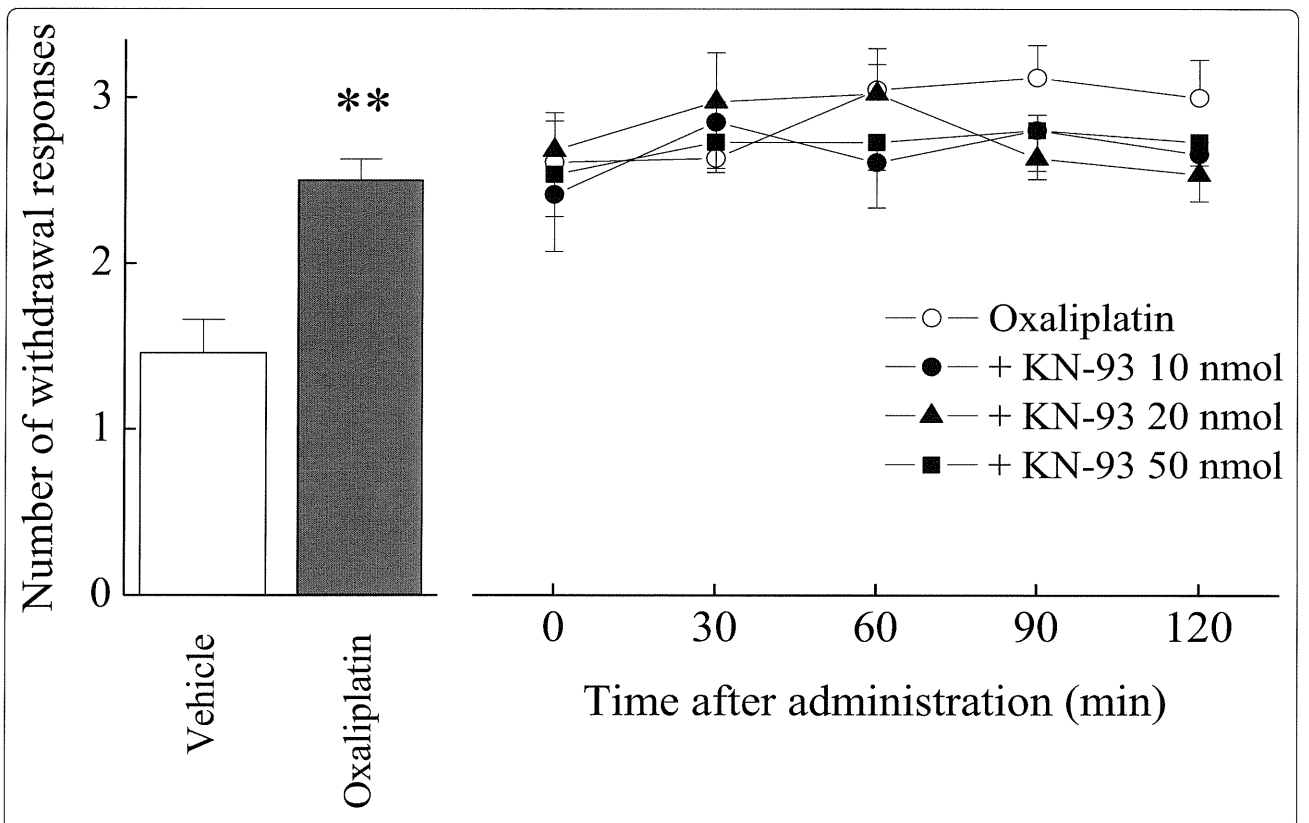

Figure 2 Effect of KN-93 on oxaliplatin-induced cold hyperalgesia in the acetone test. Rats were treated with oxaliplatin (4 mg/kg, i.p.) on days 1 and 2. We confirmed the incidence of cold hyperalgesia on day 5 and we carried out the drug evaluation on the same day. KN-93 (10-50 nmol) was administered intrathecally. The acetone test was performed immediately before (0 min) and at 30, 60, 90 and 120 min after administration. KN-93 had no effect on oxaliplatin-induced cold hyperalgesia. Values are expressed as the mean ± SEM. of 7-8 animals. $^{**}p < 0.01$ compared with vehicle (Student's *t*-test).

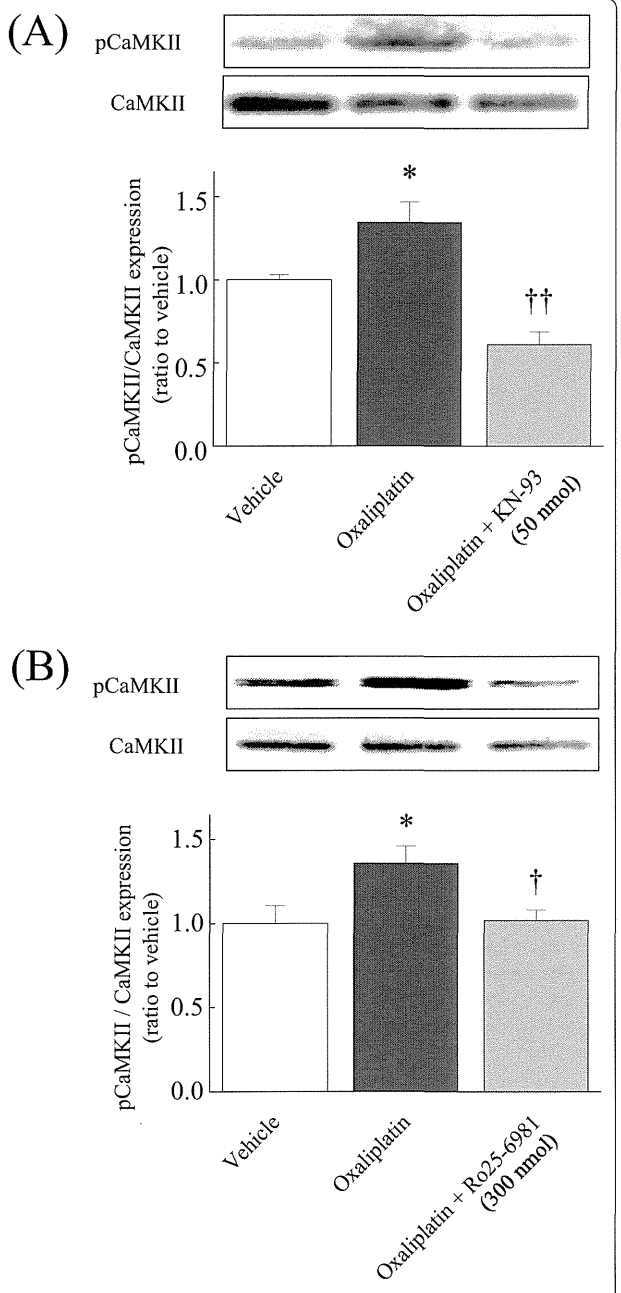

Figure 3 Effects of KN-93 and Ro 25-6981 on oxaliplatin-induced increase in spinal CaMKII phosphorylation. Rats were treated with oxaliplatin (4 mg/kg, i.p.) twice a week for 4 weeks (days 1, 2, 8, 9, 15, 16, 22 and 23). The lumbar sections (L_{4-6}) of the spinal cord were quickly removed at 30 min after administration of KN-93 (50 nmol, i.t.) or Ro 25-6981 (300 nmol, i.t.) on day 25. CaMKII phosphorylation (pCaMKII) in the lumbar sections of the spinal cord was determined by Western blotting. An increase of pCaMKII was found in the spinal cord of oxaliplatin-treated rats. Acute treatment with KN-93 (A) and Ro 25-6981 (B) reduced oxaliplatin-induced increase in the spinal pCaMKII. Values are expressed as mean ± SEM. of 6-8 animals. *$p < 0.05$ compared with vehicle, †$p < 0.05$, ††$p < 0.01$ compared with oxaliplatin alone (one-way ANOVA followed by Tukey-Kramer post-hoc test).

Effect of trifluoperazine on Oxaliplatin-induced mechanical allodynia and increase in CaMKII phosphorylation

Since trifluoperazine, an antipsychotic drug, inhibits calmodulin required for CaMKII phosphorylation [12], we tested the effect of this compound on the oxaliplatin-induced mechanical allodynia. Trifluoperazine (0.1 and 0.3 mg/kg, p.o.) significantly reduced the oxaliplatin-induced mechanical allodynia at 30 min (0.1 mg/kg: $p < 0.05$; 0.3 mg/kg: $p < 0.01$) and 120 min (0.3 mg/kg: $p < 0.05$) after the administration (Figure 4). On the other hand, trifluoperazine at the effective dose (0.3 mg/kg, p.o.) had no effect on the paw withdrawal thresholds in intact rats (Figure 5). Trifluoperazine (0.3 mg/kg, p.o.) strongly reduced the oxaliplatin-induced increase in spinal pCaMKII ($p < 0.01$, Figure 6).

Effect of trifluoperazine on motor coordination

We examined the influence of trifluoperazine on motor coordination in rota-rod test. At the time before (0 min) and 30 min after administration of trifluoperazine, there was no difference in the motor coordination among the groups-treated with distilled water, trifluoperazine 0.3 mg/kg or oxaliplatin + trifluoperazine 0.3 mg/kg (Figure 7).

Discussion

In the present study, acute treatment of the CaMKII inhibitor KN-93 reversed the oxaliplatin-induced mechanical allodynia. KN-93 binds directly to calmodulin-binding site of CaMKII and inhibits the enzyme activity [13]. KN-93 also inhibits not only CaMKII but also L-type Ca^{2+} channels [14], and therefore there is possibility that the effect of KN-93 on the mechanical allodynia depends on blockade of Ca^{2+} channels. However, its structural analog KN-92, which does not inhibit CaMKII but blocks L-type Ca^{2+} channels' current, did not reverse the oxaliplatin-induced mechanical allodynia. Moreover, the expression of pCaMKII significantly increased in the spinal cord of oxaliplatin-treated rats on day 25, and this increase of pCaMKII was blocked by KN-93. On the other hand, KN-93 had no effect on the oxaliplatin-induced cold hyperalgesia on day 5. These results indicate that the spinal CaMKII is involved in the oxaliplatin-induced mechanical allodynia but not cold hyperalgesia.

Recently, we reported that repeated administration of oxaliplatin increased the expression of NR2B protein and mRNA in the rat spinal cord on day 25 (late phase) but not on day 5 (early phase), and Ro 25-6981 reversed the oxaliplatin-induced mechanical allodynia [3], suggesting the involvement of NR2B-containing NMDA receptors. These NMDA receptors have been reported to contribute to development of spinal hyperexcitability and chronic pain [15-17]. Ca^{2+} influx through activated

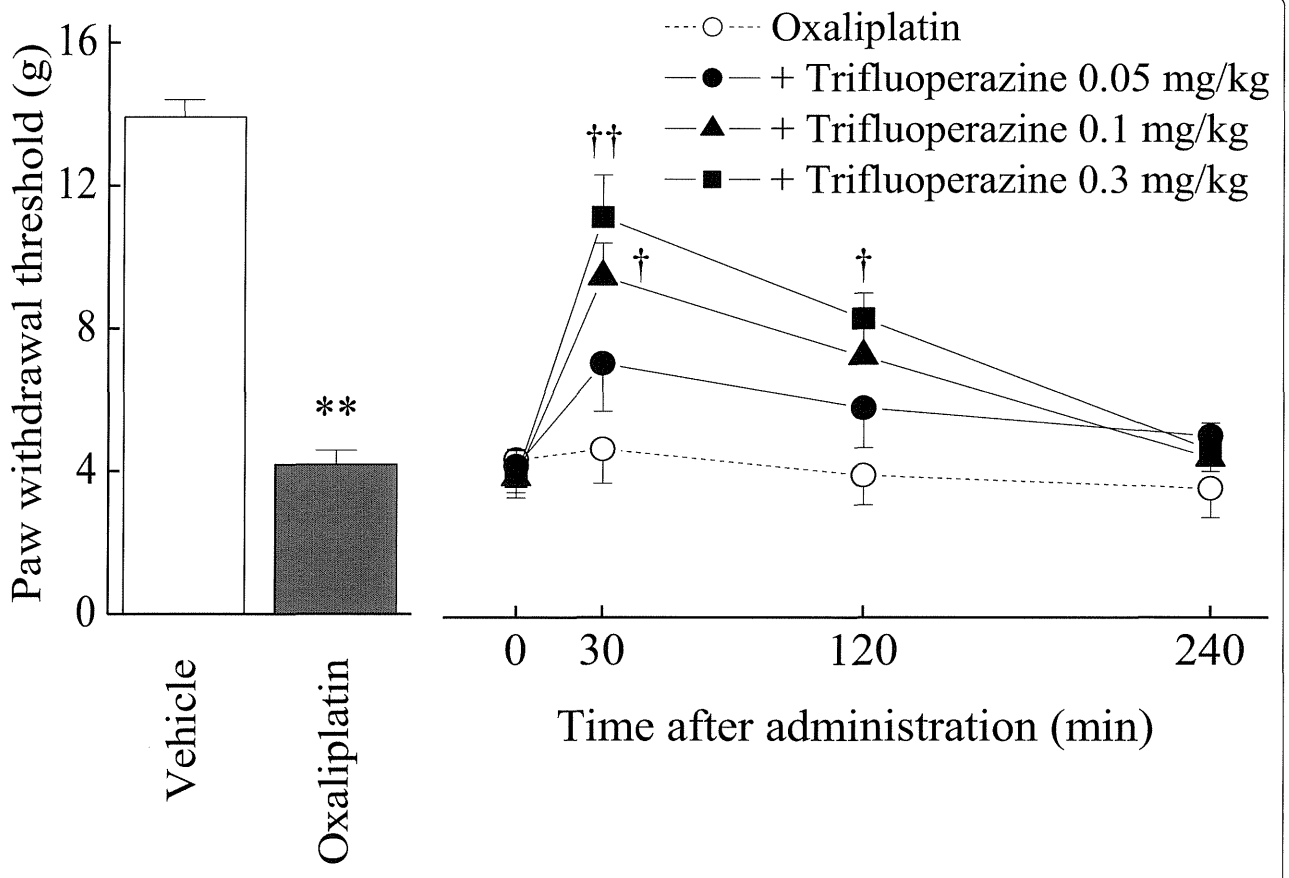

Figure 4 Effect of trifluoperazine on oxaliplatin-induced mechanical allodynia in the von Frey test. Rats were treated with oxaliplatin (4 mg/kg, i.p.) twice a week for 4 weeks (days 1, 2, 8, 9, 15, 16, 22 and 23). We confirmed the incidence of mechanical allodynia on day 24. We carried out the drug evaluation on the next day. Trifluoperazine (0.05-0.3 mg/kg) was administered orally. The von Frey test was performed immediately before (0 min) and at 30, 120 and 240 min after administration of trifluoperazine. Trifluoperazine (0.1 and 0.3 mg/kg) significantly reversed oxaliplatin-induced mechanical allodynia. Values are expressed as the mean ± SEM. of 8 animals. $^{**}p < 0.01$ compared with vehicle, $\dagger p < 0.05$, $\dagger\dagger p < 0.01$ compared with oxaliplatin alone (one-way ANOVA followed by Tukey-Kramer post-hoc test).

NMDA receptors causes multiple intracellular changes including an activation of CaMKII [18,19], and the CaMKII co-localizes with NR2B in the nociceptive regions such as the superficial dorsal horn [20]. Furthermore, NR2B mutation, which causes a lower increase in intracellular Ca^{2+} concentration by glutamate stimulation, leads to the reduction of spinal CaMKII phosphorylation in the neuropathic pain model [8,9]. In our present study, we found that the increase of pCaMKII in the spinal cord of oxaliplatin-treated rats was blocked by Ro 25-6981, the selective NR2B antagonist. Therefore, oxaliplatin may induce the CaMKII activation by increasing in the expression of NR2B-containing NMDA receptors. These findings indicate an important role of CaMKII as a downstream of NR2B-containing NMDA receptors in pain states.

In this study, we confirmed an inhibition of CaMKII phosphorylation by trifluoperazine, which inhibits calmodulin required for CaMKII activation [12]. Furthermore, we found that trifluoperazine dose-dependently reduced the oxaliplatin-induced mechanical allodynia. Previous studies showed that trifluoperazine reversed complete Freund's adjuvant-induced inflammatory pain and spinal nerve ligation-induced neuropathic pain in mice [7,11]. On the other hand, we observed that trifluoperazine at the effective dose (0.3 mg/kg) had no effect on the paw withdrawal thresholds in intact rats. In addition, trifluoperazine at the same dose did not affect the motor coordination in rota-rod test in intact and oxaliplatin-treated rats. Ye et al. [21] reported that trifluoperazine (0.125-0.5 mg/kg, i.p.) did not affect spontaneous locomotion in rats. Bhargava and Chandra [22] reported that ED50 (i.p.) of suppression of the conditioned response, an index of tranquilizing effect, is 0.58 mg/kg. Therefore, it is unlikely that the inhibitory effect of trifluoperazine on the oxaliplatin-induced pain behavior is due to its motor dysfunction or sedative effect. Taken together, the inhibitory effect of