Characterization of Acute and Chronic Neuropathies Induced by Oxaliplatin in Mice and Differential Effects of a Novel Mitochondria-targeted Antioxidant on the Neuropathies

Satoshi Toyama, M.D., Ph.D., Naohito Shimoyama, M.D., Ph.D., Yasuo Ishida, M.D., Ph.D., Takayoshi Koyasu, M.T., Hazel H. Szeto, M.D., Ph.D., Megumi Shimoyama, M.D., Ph.D.

ABSTRACT

Background: Oxaliplatin, a chemotherapeutic agent used for the treatment of colorectal cancer, induces dose-limiting neuropathy that compromises quality of life. This study aimed to reproduce, in mice, patients' symptoms of oxaliplatin-induced neuropathy and to observe effects of SS-31, a mitochondria-targeted antioxidant on the neuropathy.

Methods: Neuropathy was induced by single or repeated injections of oxaliplatin. Cold and mechanical hypersensitivities were assessed by 15°C-cold plate, temperature preference, and von Frey tests. Morphology of peripheral nerves and dorsal root ganglions, expression of spinal cord c-Fos, density of intraepidermal nerve fibers, and levels of dorsal root ganglion–reactive oxygen/nitrogen species were examined. SS-31 was administered concomitantly or after oxaliplatin injections.

Results: Single injection of oxaliplatin induced cold hypersensitivity in forepaws but not in hind paws which resolved within days (maximal forepaw shakes: 28 ± 1.5 *vs.* 9.3 ± 1.6/150 s, mean ± SEM, $P < 0.001$, n = 6 per group). Oxaliplatin-administered mice disfavored 10° and 15°C plates more than control. Paw stimulation at 15°C induced c-Fos–positive cells within superficial laminae of the dorsal horn in C7-T1 segments. Weekly administrations induced gradual development of persistent mechanical allodynia in the hind paws (minimal mechanical threshold: 0.19 ± 0.08 *vs.* 0.93 ± 0.11 g, $P < 0.001$, n = 10 per group). Microscopy revealed no overt morphological changes in peripheral nerves and dorsal root ganglions. Concomitant SS-31 administration with repeated oxaliplatin administration attenuated both cold and mechanical hypersensitivity. Decrease in intraepidermal nerve fibers and increase in dorsal root ganglion–reactive oxygen/nitrogen species were also attenuated. Acute SS-31 administration after symptoms were established reversed only cold hypersensitivity.

Conclusion: This model of oxaliplatin-induced neuropathy mimicked patients' conditions. SS-31 has potentials to prevent both acute and chronic neuropathies but is only helpful in treatment of acute neuropathy. **(ANESTHESIOLOGY 2014; 120:459-73)**

OXALIPLATIN (L-OHP) is a platinum-based chemotherapeutic agent used for the treatment of advanced colorectal cancer.[1,2] Neurotoxicity is the most common toxicity of oxaliplatin, manifesting two distinct neuropathies.[3–11] Acute neuropathy, which is common and unique to oxaliplatin, occurs acutely after administration of each oxaliplatin dose and consists of reversible cold-induced dysesthesia/paresthesia of the hands and face but rarely of the feet.[3,9] Patients experience symptoms when they touch moderately cold items that normally would not produce pain. This acute neuropathy is usually self-limiting and symptoms resolve in days, within each administration cycle. Chronic neuropathy gradually develops and intensifies after multiple administration cycles. Patients complain of ongoing dysesthesia/paresthesia of extremities and impaired sensorymotor coordination may also be present. The chronic neuropathy is similar to those seen with other platinum derivatives, and once developed, most patients continue to experience symptoms for months. Such side effects can seriously compromise patients' quality of life and lead to changes in treatment to nonneurotoxic agents with obvious negative implications for disease outcomes.

What We Already Know about This Topic

- Oxaliplatin and other platinum-containing chemotherapeutics often cause a form of peripheral neuropathy reducing quality of life and limiting the dose of drug which can be tolerated

What This Article Tells Us That Is New

- In mice, oxaliplatin causes sensitization to both cold and mechanical stimuli, most prominently in the forepaws
- Administration of the antioxidant agent SS-31 reduced both the nociceptive sensitization and the accumulation of reactive oxygen species

Recently, multiple animal studies have been described that use various doses of oxaliplatin with various administration schedules. Some of these studies report that oxaliplatin

The work was presented in part at the 14th World Congress on Pain, Milan, Italy, August 28, 2012.

Submitted for publication February 13, 2013. Accepted for publication August 22, 2013. From the Department of Anesthesiology (S.T., N.S., M.S.) and Department of Pathology (Y.I., T.K.), Teikyo University Chiba Medical Center, Ichihara, Chiba, Japan; Division of Palliative Medicine, Department of Anesthesiology (N.S.), Jikei Medical University Hospital, Tokyo, Japan; and Department of Pharmacology (H.H.S.), Weill Cornell Medical College, New York, New York.

 Anesthesiology 2014; 120:459-73

causes damage to cell bodies,[12–15] alterations in nucleolus morphology,[12] selective atrophy of subpopulation of dorsal root ganglion (DRG) neurons,[16] and degeneration of peripheral nerve axons.[12,17] However, different investigators have failed to show axonal degeneration.[18] Because pathological changes may depend on doses and schedule of oxaliplatin administration,[12,14] it is essential that the doses and schedule of administration in animal models used to study the neuropathies will produce the same condition as those in patients. Thus, the doses used should produce antitumor effects and the administration schedule should include rest periods that allow recovery from acute damage. The aim of this study was to develop such an animal model in mice and to investigate its neuropathic symptoms in detail so as to compare them with symptoms observed in patients. We used doses that had been reported to produce antitumor effects[19–21] and set the schedule that would allow a significant degree of recovery from acute neuropathy within the cycle. We also confirmed the antitumor effects of the doses in mice given by our study schedule. Furthermore, using this model, we attempted to reveal possible morphological changes in the peripheral nerve and/or DRG neurons that may have relevance to the mechanism of the neuropathic symptoms. In addition, in wake of recent reports by other investigators that show the involvement of mitochondrial dysfunction and oxidative stress in chronic oxaliplatin-induced neuropathy,[18,22–25] we investigated the effects of SS-31, a novel mitochondria-targeted antioxidant, in this model. SS-31 is a tetrapeptide that targets and concentrates on the inner mitochondrial membrane, the site of electron transport chain, and reactive oxygen species (ROS) production.[26,27] SS-31 has been demonstrated to be highly effective in several animal disease models associated with mitochondrial dysfunction and oxidative stress.[28–34] We examined whether SS-31 can protect against the development of acute and chronic neuropathies and also whether it can alleviate the symptoms after each neuropathy has been established.

Materials and Methods

Experiments were approved by the Institutional Animal Use Committee of Teikyo University, Tokyo, Japan, and were conducted in accordance with the National Institutes of Health guidelines and the International Association for the Study of Pain Committee for Research and Ethical Issue guidelines for animal research.[35] The experimenter was blinded to treatment in all behavioral experiments. The experimenter who performed the quantification in the morphological studies of peripheral nerves and DRGs was not blinded to treatment. In all other morphological quantifications, the experimenter was blinded to treatment.

Animals, Drugs, and Cell Line

Male mice, 9 weeks old at the time of first drug administration, were used in the experiments. BALB/c mice were used except for the experiment on antitumor effects of oxaliplatin in which SCID mice were used. All mice were housed on a 12:12 h dark–light cycle with food and water *ad libitum*. Oxaliplatin was obtained from Yakult Co., Ltd. (Tokyo, Japan). Oxaliplatin was dissolved in distillated water and was injected intraperitoneally in a volume of 0.1 ml/10 g mouse weight. SS-31 (H-D-Arg-2',6'dimethylTyr-Lys-Phe-NH_2) was obtained from Stealth Peptides, Inc. (Newton Centre, MA). SS-31 was dissolved in normal saline for continuous subcutaneous administration at a rate of 0.1 μl/h or in a volume of 0.1 ml/10 g mouse weight as subcutaneous injections. A human colorectal cell line (HCT116) was a generous gift from Dr. Soichiro Murata, M.D., Ph.D. (Associate Professor, Department of Surgery, Tsukuba University Graduate School of Medicine,Tsukuba, Ibaraki, Japan). The cancer cell line was subdivided in multiple tubes for stock in liquid nitrogen immediately after possession. Stock cultures were grown in high-glucose Dulbecco Modified Eagle Medium containing 10% fetal bovine serum and 1% antibiotics. Cells were grown in growth medium at 37°C in a 95% air–5% CO_2 humidified incubator.

Acute Neuropathy

Nocifensive Response to Moderately Cold Temperature.

To observe the development of nocifensive response to moderately cold temperature induced by oxaliplatin, we first examined paw-shaking behavior of mice placed on a cold plate (LHP-1700CP; TECA, Chicago, IL) set at 15°C (15°C-cold plate test) and studied the effects of different doses of oxaliplatin and the time courses of the effects after a single injection. The number of forepaw and hind paw shakes was counted during a 150-s test period while mice were placed on the cold plate (15°C-cold plate test). Testing was performed before any drug administration and 1, 2, 3, 4, 5, 7, 9, and 12 days after oxaliplatin (2, 5, or 15 mg/kg) or vehicle administration. We used the cold plate test in our study because it enables us to examine the response to a specific temperature. Paw-shaking behavior has been accepted as a nocifensive behavior in multiple pain tests in mice. Paw-shaking behavior is used as a parameter in the acetone test[36] and is also an end point of the hot plate test.[37] However, in the hot or cold plate test, if the animal stands by its hind limbs, the forepaws would not be stimulated by the plate. Thus in our study, to ensure that both forepaws and hind paws were stimulated by the cold plate, we placed a clear plastic cover with ventilation pores over the cold plate to produce a ceiling 3.5 cm from the cold plate so that mice would not be able to stand by their hind limbs and would place both forelimbs and hind limbs on the cold plate. Furthermore, to support the behavioral results obtained by the 15°C-cold plate test, c-Fos expressions in the spinal dorsal horn at the thoracic and lumbar levels were examined in mice whose forepaws and hind paws were stimulated by the cold plate set at 15°C (see Materials and Methods section,

Acute Neuropathy, c-Fos Expression in the Dorsal Horn after Moderately Cold Thermal Stimulation of the Paws in Oxaliplatin-administered Mice).

Temperature Preference. Change in temperature preference induced by a single injection of oxaliplatin was assessed by the two-plate temperature preference test.[38] Each mouse was placed in a chamber containing two identical, adjacent floor platforms (LHP-1700CP; TECA) with one set to a fixed temperature of 24°C (room temperature) and the other (test plate) set to one of the following different temperatures: 24°, 20°, 15°, 10°, 5°, or 0°C. Mice were free to explore, and the time spent on the test plate during a 10-min test period was measured. Testing was performed before any drug administration and 4 days after the administration of oxaliplatin (15 mg/kg), which was the time of peak effect determined from the above experiment that examined the time course of cold hypersensitivity. The 15 mg/kg dose of oxaliplatin was selected because it was an effective dose in the above experiment.

Effect of Weekly Repeated Administrations of Oxaliplatin on Cold Hypersensitivity. Oxaliplatin at 15 mg/kg was injected weekly for three cycles, and cold hypersensitivity was assessed 4 days after each injection by the number of paw shakes in the 15°C-cold plate test described above (see Materials and Methods section, *Acute Neuropathy*, Nocifensive Response to Moderately Cold Temperature).

c-Fos Expression in the Dorsal Horn after Moderately Cold Thermal Stimulation of the Paws in Oxaliplatin-administered Mice. To observe the activation of dorsal horn neurons by moderately cold thermal stimulation in oxaliplatin-administered mice, c-Fos expression in the spinal dorsal horn was examined.[39] Mice were given oxaliplatin (15 mg/kg) or vehicle, and 4 days later, 15°C thermal stimulations were applied to the paws. By using the cold plate set at 15°C, 10 repetitive stimulations lasting 30 s each were applied to the right forepaw and hind paw in 2-min cycles over a total period of 20 min.[38] Two hours after the application, each mouse was deeply anesthetized with pentobarbital and perfused intracardially with saline followed by a fixative solution which contained 4% paraformaldehyde in 0.01 M phosphate buffer saline (PBS). The C7-T1 (input from the forepaws) and L4-5 (input from the hind paws) segments of the spinal cord were excised for immunohistochemical studies. The excised spinal cord segments were processed and sliced by methods reported previously.[40] Antibodies against c-Fos (rabbit, polyclonal; Oncogene Research Products, San Diego, CA) were used. The sections were observed under a fluorescence microscope. The number of Fos-like immunoreactive nuclei in the superficial laminae of the ipsilateral dorsal horn in three random sections per segment was counted and averaged.

Chronic Neuropathy

Mechanical threshold to induce paw withdrawal responses was assessed by stimulation with von Frey hairs (Semmes-Weinstein Monofilaments; Stoelting Co., Wood Dale, IL). Mice were placed in a clear plastic chamber with a wire mesh floor, which provided full access to the planter surface of the hind paws. Mice were allowed to habituate for at least 30 min before testing. The paws were touched with one of a series of nine von Frey filaments with logarithmically incremental stiffness (0.023–3.630 g) starting with the filament of 0.407 g. The 50% mechanical withdrawal thresholds were determined using the up–down method described by Chaplan *et al.*[41]

Effect of a Single Dose of Oxaliplatin on Mechanical Threshold. Mechanical thresholds were determined before and 4, 7, and 12 days after a single injection of oxaliplatin (15 mg/kg) or vehicle.

Effect of Weekly Repeated Administrations of Oxaliplatin on Mechanical Threshold. Oxaliplatin at 15 mg/kg or vehicle was injected weekly for three cycles, and mechanical threshold was assessed 4 days after each injection and weekly thereafter for 5 more weeks. In other mice, oxaliplatin at 5 or 10 mg/kg or vehicle was injected weekly for nine cycles, and mechanical threshold was assessed 4 days after each injection.

Fos-like Immunoreactivity of Dorsal Horn Neurons in Repeated Oxaliplatin-administered Mice. To observe whether dorsal horn neurons were activated after multiple weekly oxaliplatin administrations, some of the mice that were given nine weekly injections of oxaliplatin (each dose at 10 mg/kg) or vehicle in the above study were sacrificed for c-Fos immunohistochemical studies. Fos-like immunostaining as described above was performed in L4-5 segments of the spinal dorsal cord excised from the mice 4 days after the last injection.

Morphological Studies of Peripheral Nerves and DRGs

Some of the mice that underwent behavioral testing after repeated administrations of oxaliplatin were sacrificed for light and electron microscopic studies on the last day of testing. Each mouse was deeply anesthetized with pentobarbital and perfused intracardially with saline followed by a fixative solution which contained 2% glutaraldehyde. Portions of the right brachial and sciatic nerve and C8, T1, L4, and L5 DRGs were excised for light and electron microscopy. For light microscopy, semithin sections (1 μm) were stained with toluidine blue. For electron microscopy, the excised tissues were postfixed for 45 min (15 min × 3) with 1% osmium tetroxide dissolved in PBS, dehydrated through a graded series of ethanol, cleared by QY-1, and then embedded in an epoxy resin mixture. Ultrathin sections (100 nm) were cut with a diamond knife. The number of myelinated and unmyelinated fibers of the sciatic nerve section taken just distal to the point where the posterior biceps semitendinosus nerve branches off was counted. The areas of nucleoli, nuclei, and somata of L4 DRG neurons were measured, and the values were averaged for each mouse.

Effects of SS-31 Treatment on Oxaliplatin-induced Neuropathy

Effects of Continuous Administration of SS-31 during Repeated Oxaliplatin Administrations. SS-31 at 5 $mg \cdot kg^{-1} \cdot day^{-1}$ or vehicle was continuously administered *via* subcutaneously implanted Alzet 1004 Micro-Osmotic Pumps (Alzet, Cupertino, CA). The pump was implanted 2 days before the first oxaliplatin injection to allow the infusion rate of the pump to stabilize. Mice were given three weekly administrations of oxaliplatin (15 mg/kg) and were tested 4 days after each injection for cold hypersensitivity and mechanical allodynia by the 15°C-cold plate test and the von Frey hair test, respectively (see Materials and Methods section, *Acute Neuropathy*, Nocifensive Response to Moderately Cold Temperature and *Chronic Neuropathy*).

Other mice implanted with Alzet pumps filled with SS-31 or vehicle and given repeated administrations of oxaliplatin as above were sacrificed on day 18 for ROS/reactive nitrogen species (RNS) assay of lumbar DRGs and intraepidermal nerve fiber (IENF) examination (see Materials and Methods section, *ROS/RNS Assays of DRGs* and *Quantification of IENFs*).

Effects of Acute Administration of SS-31 on Established Oxaliplatin-induced Neuropathic Symptoms and Dorsal Horn Neuron Activation. Mice were given a single dose of oxaliplatin (15 mg/kg). Four days after the injection, they were tested for cold allodynia by 15°C-cold plate test before and 1, 2, 4, and 6 h after the subcutaneous administration of SS-31 (10 mg/kg) or vehicle. Other mice were given three weekly injections of oxaliplatin (each dose at 15 mg/kg), and 4 days after the third injection, mechanical thresholds were measured by the von Frey hair test before and 2 h after the subcutaneous administration of SS-31 or vehicle.

In addition, the effect of acute administration of SS-31 on the activation of dorsal horn neurons by moderately cold thermal stimulation in oxaliplatin-administered mice was examined by c-Fos immunohistochemistry of the spinal cord. Mice were given a single dose of oxaliplatin (15 mg/kg). On day 4, SS-31 (10 mg/kg) or saline was given subcutaneously, and 2 h later, 15°C thermal stimulations were applied to the paws as the above experiment. Fos-like immunostaining was performed in C7-T1 segments of the spinal cord as described above (see Materials and Methods section, *Acute Neuropathy*, c-Fos Expression in the Dorsal Horn after Moderately Cold Thermal Stimulation of the Paws in Oxaliplatin-administered Mice).

ROS/RNS Assays of DRGs

Lumbar DRGs were excised from mice anesthetized with sevoflurane and homogenized in PBS. Homogenates were subjected to centrifugation at 10,000*g* for 5 min to remove insoluble particles. The concentration of ROS and RNS liberated was measured using Oxiselect *in vitro* ROS/RNS assay kit (Cell Biolabs, Inc., San Diego, CA) according to the manufacturer's instructions. The relative fluorescence of the samples and the standards were read at 480 nm excitation/530 nm emissions using a multilabel plate reader (Arvo, PerkinElmer, Yokohama, Japan). The ROS/RNS liberated by DRGs were calculated using a 2',7'-dichlorodihydrofluorescein standard curve. Data were normalized to one DRG level and were expressed as percentage of the mean value of the control group.

Quantification of IENFs

Plantar skin specimens were excised from the hind paw between the calcaneous and the digital tori. The specimens were immediately soaked in 4% paraformaldehyde for overnight fixation and then transferred to 30% sucrose and left overnight at 4°C. The specimens were then embedded in TissueTek OCT compound (Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands) and frozen and sliced on a cryostat into 25 μm sections. The sections were collected in PBS and processed by a free-floating protocol. The sections were incubated in a blocking solution that consisted of 0.5% normal goat serum and 0.3% Triton-X100 in PBS for 30 min at room temperature. They were then incubated overnight at 4°C in primary antibodies in the blocking solution. Antibodies against protein gene-product 9.5 (rabbit, affinity purified; Enzo Life Sciences, Farmingdale, NY) was used. Following this step, the sections were incubated in biotinylated goat anti-rabbit IgG (Vector Laboratories, Belmont, CA) solution for 90 min at room temperature and then incubated with Alexa Fluor 488 streptavidin conjugate (Molecular Probes, Eugene, OR) for 60 min at room temperature. Between each step, the sections were rinsed with PBS three times. The sections were observed under a fluorescence microscope. The number of IENFs per millimeter of the epidermal border was counted in three random sections and averaged for each mouse.

Antitumor Effect of Oxaliplatin

HCT116 cells (1×10^6) were implanted subcutaneously into the right thighs of SCID mice. Ten days after implantation, mice were randomly divided into five groups and were given three weekly administrations of oxaliplatin doses at 2, 5, 10, or 15 mg/kg or vehicle. Four days after the third injection, the mice were sacrificed and the tumors were excised and weighed.

Statistical Analysis

Statistical analyses were carried out with SigmaPlot statistical software package for Windows (version 11.0; Systat, San Jose, CA). Data were analyzed using a one-way or two-way ANOVA for repeated measures followed by the Bonferroni-corrected *t* test or paired *t* test, where appropriate. A two-tailed *P* value less than 0.05 was considered significant. *Post hoc* testing was only reported when statistically significant main effects were observed. There were no missing data for all analyses.

Results

Acute Neuropathy

Nocifensive Response to Moderately Cold Temperature. In mice given a single injection of oxaliplatin at 15 mg/kg, the number of forepaw shakes in the 15°C-cold plate test significantly increased compared with control mice by 2 days after injection. The effect peaked 4 days after injection and returned to control level by 9 days after injection (fig. 1A). None of the mice presented shaking, lifting, or licking of the hind paws. Single injection of oxaliplatin at 2 and 5 mg/kg did not have any effects in the 15°C-cold plate test.

Temperature Preference. In the temperature preference test, when the two plates were set at the same temperature (24°C), neither oxaliplatin- (15 mg/kg) nor vehicle-administered mice displayed any preference. When the test plate was cooled to 20°C or below while the adjacent plate's temperature was fixed to 24°C, both oxaliplatin- and vehicle-administered mice showed a preference for the warmer plate. The preference was not different between the two groups when the test plate was set at 20°C, but when it was set at 15° or 10°C, the oxaliplatin-administered mice showed a greater preference to the warmer plate compared with the vehicle-administered mice (fig. 1B). No difference in preference was present when the test plate's temperature was set below 10°C.

Effect of Weekly Repeated Administrations of Oxaliplatin on Cold Hypersensitivity. The effect of three weekly administrations of oxaliplatin (each dose at 15 mg/kg) on the 15°C-cold plate test is shown in figure 1C. In the oxaliplatin-administered mice, the number of forepaw shakes significantly increased compared with the vehicle-administered mice when tested 4 days after each oxaliplatin injection. The number of forepaw shakes did not further increase by repeating the oxaliplatin injections (day 4 *vs.* day 11: $P = 1.000$; day 4 *vs.* day 18, $P = 0.120$; day 11 *vs.* day 18, $P = 0.216$). The number of forepaw shakes significantly decreased 1 week later (day 18 *vs.* day 25: $P < 0.001$). Mice presented no or very few shaking and no lifting or licking of the hind paws during any of the 15°C-cold plate tests performed during the course of the experiment (fig. 1C).

Fos-like Immunoreactivity of Dorsal Horn Neurons after 15°C Thermal Stimulation of the Paws. Spinal cord segments were harvested from the oxaliplatin-administered mice after thermal stimulation of paws by a cold plate set at 15°C. In the spinal cord slices of C7-T1 segments, many Fos-like immunoreactive cells were present in the superficial laminae, and some also in intermediate and deep laminae of the dorsal horn (fig. 2A). In the L4-5 segments, no or very few Fos-like immunoreactive cells were present in the superficial laminae, and some Fos-like immunoreactive cells were seen in the deep laminae (fig. 2B). In vehicle-administered mice, no or very few Fos-like immunoreactive cells were present in the dorsal horn of spinal cord slices of either the C7-T1 segments (fig. 2C) or the L4-5 segments. The number of Fos-like immunoreactive cells in the superficial dorsal horn of the C7-T1 segments was significantly greater in oxaliplatin-administered mice as compared with vehicle-administered mice (fig. 2D). This difference was not present in the L4-5 segments (fig. 2D)

Chronic Neuropathy

Effect of Weekly Repeated Administrations of Oxaliplatin on Mechanical Threshold. No change in mechanical threshold was seen after a single dose of oxaliplatin (15 mg/kg; fig. 3A). However, in mice given three weekly administrations of oxaliplatin (each dose at 15 mg/kg), mice showed a decrease in mechanical threshold after the second and third doses of oxaliplatin as compared with control (fig. 3B). The effect started to gradually diminish 3 weeks after the last dose but was still present after 5 weeks (fig. 3B).

The effects of weekly administrations of lower oxaliplatin doses (5 and 10 mg/kg) on mechanical threshold are shown in figure 3C. Nine weekly administrations of 5 mg/kg doses (cumulative dose of 45 mg/kg) induced no significant decrease in mechanical threshold compared with vehicle administration. In animals given nine weekly administrations of 10 mg/kg doses, mechanical allodynia developed gradually. After four doses (cumulative dose of 40 mg/kg), the level of mechanical allodynia was similar to that after three doses of 15 mg/kg (cumulative dose of 45 mg/kg) and further progressed with additional doses (fig. 3C).

Fos-like Immunoreactivity of Dorsal Horn Neurons in Repeated Oxaliplatin-administered Mice. The L4-5 segments of the spinal cord were harvested from mice after nine weekly administrations of 10 mg/kg doses of oxaliplatin or vehicle. Fos-like immunoreactive cells were not present in the dorsal horn of the spinal cord in either oxaliplatin- or vehicle-administered mice.

Morphological Studies of Peripheral Nerves and DRGs

Microscopic examinations of the sciatic and brachial nerves and C8, T1, L4, and L5 DRGs harvested after three weekly administrations of 15 mg/kg doses of oxaliplatin or nine weekly administrations of 10 mg/kg doses of oxaliplatin revealed no overt morphological changes compared with vehicle administration (figs. 4 and 5). No difference in the number of myelinated and unmyelinated fibers of the sciatic nerve in vehicle-administered and oxaliplatin-administered mice was present (fig. 6A). Morphometric analysis of DRG neurons also revealed no difference between vehicle-administered and oxaliplatin-administered mice (fig. 6B). No morphological changes in Schwann cells and mitochondria within the nerve fibers and DRG neurons could be detected. Vacuolation of some mitochondria in peripheral nerve axons were observed in both vehicle- and oxaliplatin-treated mice. In contrast, significant loss of IENFs of the plantar skin was observed after repeated administrations of oxaliplatin (fig. 7).

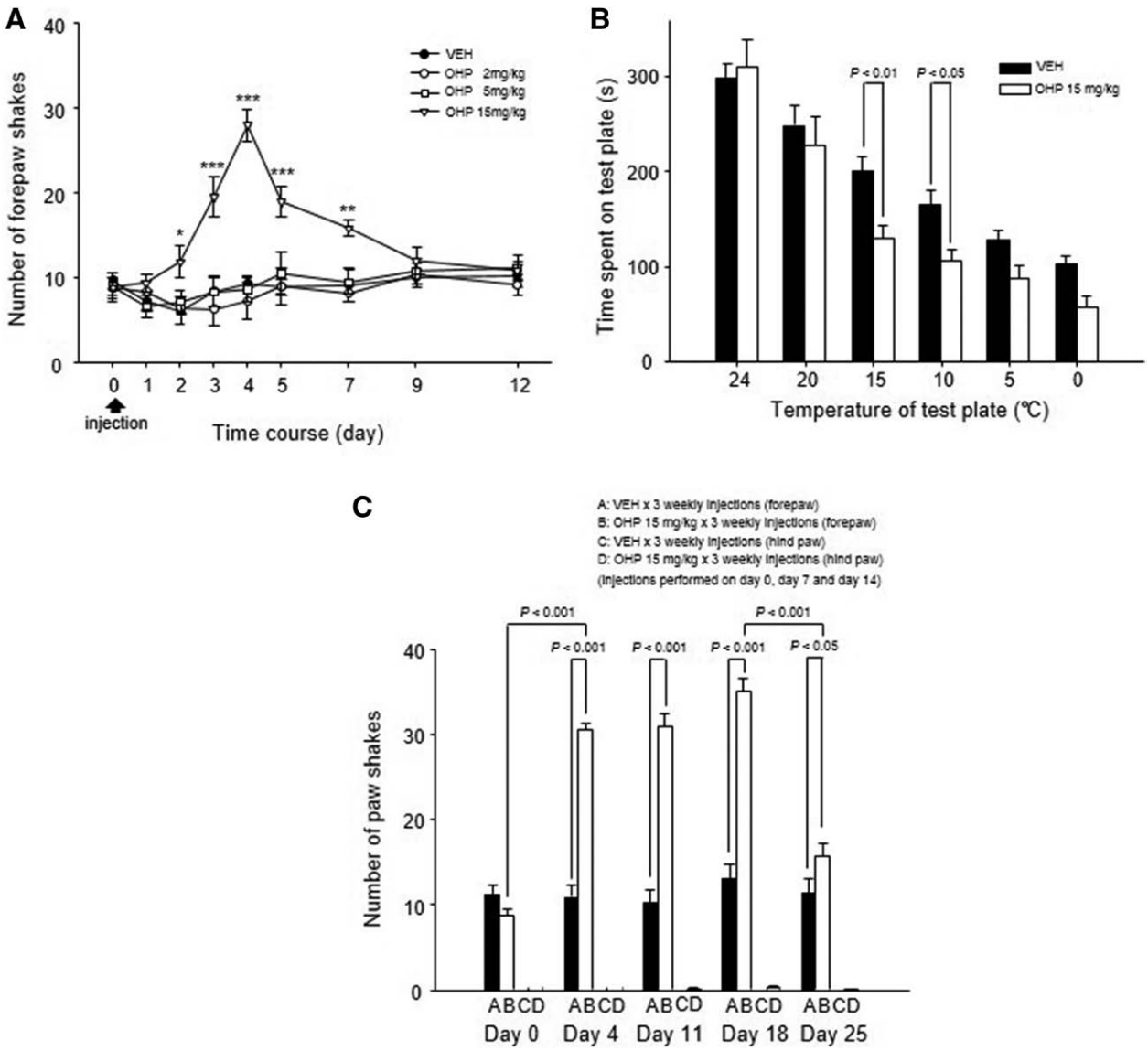

Fig. 1. Cold hypersensitivity after administration of oxaliplatin to mice. (*A*) Cold hypersensitivity was assessed by counting forepaw and hind paw shakes of mice placed on a cold plate set at 15°C during 150-s testing periods (15°C-cold plate test). Assessments were made before and 1, 2, 3, 4, 5, 7, 9, and 12 days after a single injection of oxaliplatin (2, 5, or 15 mg/kg) or vehicle (n = 6 for each group). (*B*) Temperature preference was assessed by the two-plate temperature preference test. Mice were placed in a chamber containing two identical, adjacent floor platforms with one set to a fixed temperature of 24°C and the other (test plate) set to 24°, 20°, 15°, 5°, or 0°C. The time spent on the test plate during a 10-min testing period was measured. Testing was performed before any drug administration and 4 days after administration of oxaliplatin (15 mg/kg) or vehicle (n = 10 for each group). (*C*) Cold hypersensitivity was also assessed by the 15°C-cold plate test 4 days after each of the three weekly administrations of oxaliplatin (each dose at 15 mg/kg; n = 10 for each group). Data were given as the mean ± SEM and analyzed by two-way ANOVA for repeated measures in (*A*) and (*C*) and by two-way ANOVA in (*B*) followed by Bonferroni multiple comparison tests (*A*, group effect: $P < 0.001$; time effect: $P < 0.001$; *B*, group effect: $P < 0.001$; temperature effect: $P < 0.001$; *C*, group effect: $P < 0.001$; time effect: $P < 0.001$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ *vs.* corresponding time point of control group (*A*). OHP = oxaliplatin; VEH = vehicle.

Effects of SS-31 Treatment on Oxaliplatin-induced Neuropathy

Continuous Administration of SS-31 throughout Oxaliplatin Treatment. Continuous administration of SS-31 throughout the course of three weekly administrations of oxaliplatin decreased the number of forepaw shakes compared with control in the 15°C-cold plate test performed 4 days after each of the three weekly injections of oxaliplatin (fig. 8A). Continuous SS-31 treatment also attenuated the decrease in mechanical threshold compared with control when tested 4 days after the second and third injections of oxaliplatin (fig. 8B). Loss

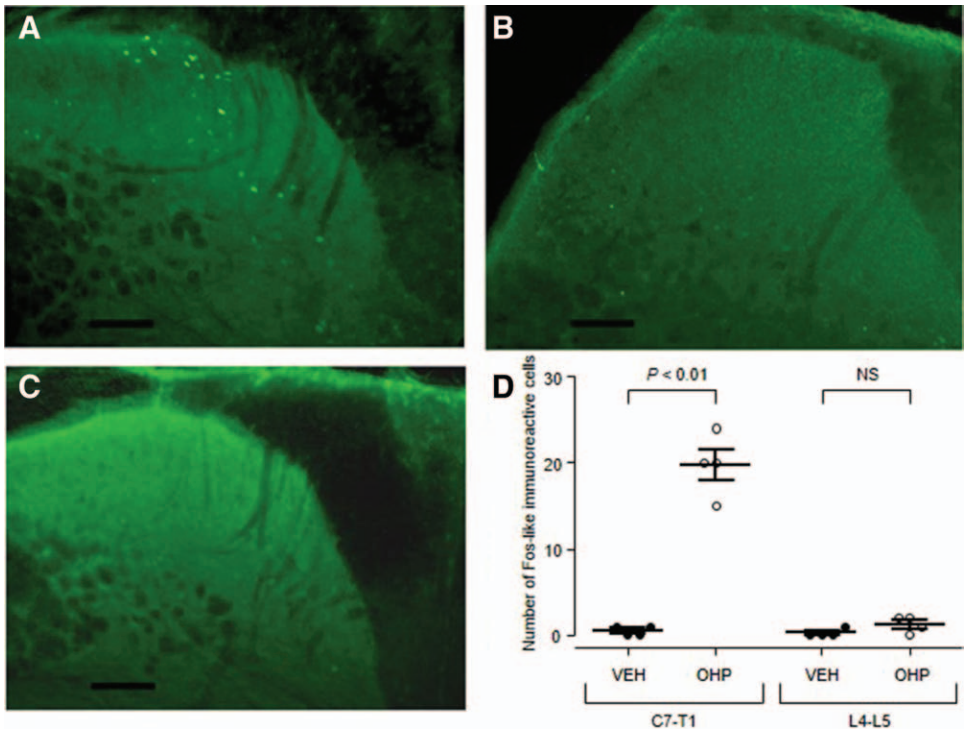

Fig. 2. c-Fos immunostaining of the spinal dorsal horn in sections from C7-T1 and L4-5 segments of the spinal cord after a moderately cold thermal stimulation of 15°C to paws of mice on day 4 postoxaliplatin (15 mg/kg) or vehicle administration. Ten repetitive stimulation lasting 30 s each were applied to the forepaws and hind paws in 2-min cycles over a total period of 20 min and mice were sacrificed after 2 h. Sections from C7-T1 and L4-5 segments of the spinal cord in an oxaliplatin-administered mouse (*A* and *B*, respectively) and section from C7-T1 segment of the spinal cord in vehicle-administered mouse (*C*). *Scale bars* are 50 μm. (*D*) Counts of Fos-like immunoreactive cells in the superficial laminae. The number of Fos-like immunoreactive nuclei in the superficial laminae of the ipsilateral dorsal horn in three random sections per segment was counted and averaged. Data were given as scatter plots of the average number of the Fos-like immunoreactive cells in vehicle- and oxaliplatin-administered mice with mean ± SEM, and analyzed by two-tailed paired *t* test (n = 4 for each group). NS = not significant; OHP = oxaliplatin; VEH = vehicle.

of IENFs induced by chronic oxaliplatin administration was also mitigated by continuous SS-31 treatment (fig. 7, C and D).

Change in ROS/RNS Levels in DRGs. Total ROS/RNS released from lumbar DRGs in mice treated with three weekly administrations of oxaliplatin doses (15 mg/kg) were significantly increased compared with control. This increase was significantly attenuated when mice received continuous SS-31 treatment concomitantly (fig. 9).

Acute Administration of SS-31 after Development of Symptoms. Acute administration of SS-31 in mice with cold hypersensitivity induced by a single injection of oxaliplatin resulted in alleviation of cold hypersensitivity tested by the 15°C-cold plate test at 1 h after SS-31 administration. The effect was still present at 4 h after administration (fig. 10). Acute administration of SS-31 also inhibited the expression of c-Fos in the superficial laminae of the dorsal horn in C7-T1 segments of the spinal cord induced by 15°C thermal stimulation in mice given a single injection of oxaliplatin (fig. 11). No effect on mechanical allodynia induced by three weekly injections of oxaliplatin was observed by acute SS-31 administration (fig. 12).

Antitumor Effect of Oxaliplatin in Mice

The weight of tumors in mice treated with three weekly doses of oxaliplatin at 5, 10, and 15 mg/kg doses (0.71 ± 0.13 g, 0.62 ± 0.25 g, and 0.48 ± 0.30 g, respectively, in mean ± SEM, n = 5 for each group) was significantly smaller than weight of tumors in mice treated with vehicle (1.71 ± 0.33 g, mean ± SEM, n = 5). Analysis was performed by a one-way ANOVA ($P = 0.013$) followed by Bonferroni multiple comparison tests ($P < 0.05$ for each comparison). The weight of tumors in mice treated with three weekly doses of oxaliplatin at 2 mg/kg (1.25 ± 0.19 g, mean ± SEM, n = 5) was not significantly different from control ($P = 0.222$).

General Toxicity

Single administration of oxaliplatin (15 mg/kg) induced a decrease in body weight compared with vehicle administration from day 2 to day 9 ($P < 0.05$). Three weekly administrations of 15 mg/kg doses (cumulative dose of 45 mg/kg) resulted in less weight gain compared with vehicle administration throughout the experiment ($P < 0.01$). The weight recovered after the cessation of oxaliplatin administration. Multiple weekly administrations of

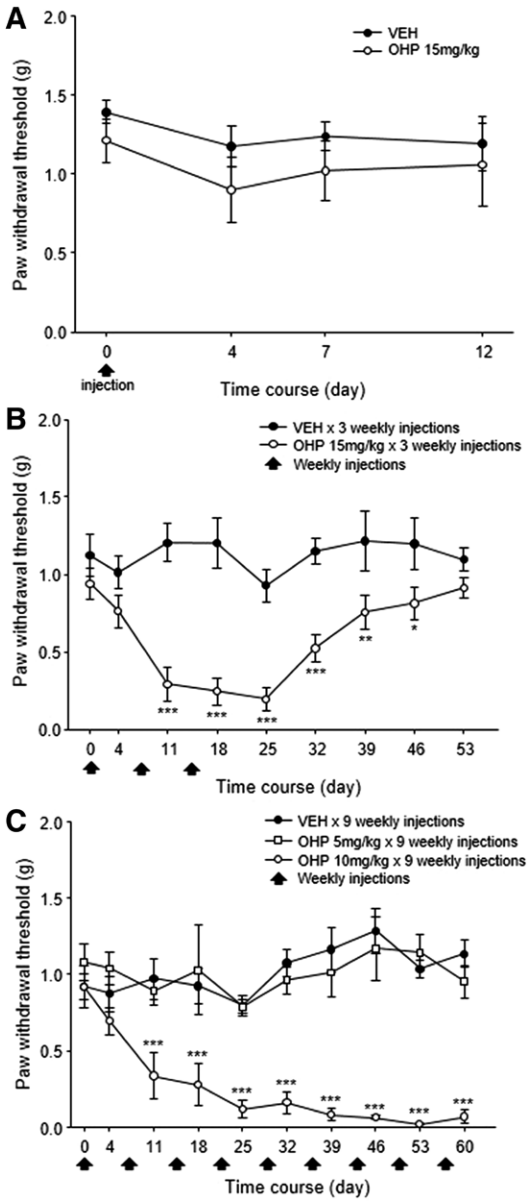

Fig. 3. Mechanical thresholds in oxaliplatin-administered mice. Assessment was made by stimulation with von Frey filaments. The 50% mechanical withdrawal thresholds were determined using the up–down method (Chaplan *et al.*[41]). (*A*) Mechanical thresholds were determined before and 4, 7, and 12 days after a single injection of oxaliplatin (15 mg/kg) or vehicle (n = 10 for each group). (*B*) Oxaliplatin at 15 mg/kg or vehicle was injected weekly for three cycles, and mechanical thresholds were assessed 4 days after each injection and thereafter weekly for 5 more weeks (n = 10 for each group). (*C*) Oxaliplatin at 5 or 10 mg/kg or vehicle was injected weekly for nine cycles, and mechanical thresholds were assessed 4 days after each injection (n = 6 for each group). Data were given as the mean ± SEM and analyzed by two-way ANOVA for repeated measures (*A*, group effect: $P = 0.326$; time effect: $P = 0.143$; *B*, group effect: $P < 0.001$; time effect: $P < 0.001$; *C*, group effect: $P < 0.001$; time effect: $P = 0.003$) followed by Bonferroni multiple comparison tests where the main effects were significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ *vs.* corresponding time point of control group. OHP = oxaliplatin; VEH = vehicle.

5 and 10 mg/kg doses (cumulative doses of 45 and 90 mg/kg, respectively) did not result in any significant differences in weight gain compared with vehicle administration. There were no deaths in the oxaliplatin-administered mice irrespective of the dose used during the course of the experiments.

Discussion

In patients, acute neuropathy occurs abruptly after administration of oxaliplatin. Patients experience dysesthesia/paresthesia when they touch moderately cold items that normally would not produce pain.[3,7,8] The symptom occurs in the hands and face and rarely in the feet.[3,9] It diminishes during the rest period of each cycle and recurs with subsequent administrations.[6] Single oxaliplatin administration to mice resulted in increased sensitivity to moderately cold temperature demonstrated by increased nocifensive behavior in response to 15°C stimulation. In naïve mice, the response peaked at 4 days and markedly diminished by 1 week after administration. With weekly administrations, cold hypersensitivity developed after each injection, but the intensity did not increase with repeated administrations, and the symptom quickly diminished after cessation of administration. The cold-induced behavior was present only in forepaws and not in hind paws. This was confirmed by examination of c-Fos expression in the dorsal horn. Cold stimulation (15°C) to forepaws and hind paws of oxaliplatin-treated mice invoked remarkable c-Fos expression in superficial laminae only in C7-T1 and not in L4-5 segments of the spinal cord. The distribution of c-Fos expression was in agreement with the localization of neurons receiving noxious inputs, and taken together with the behavior results, it suggests that the forepaws but not hind paws received noxious stimulation by 15°C stimulation. Noxious cold stimulation has been shown to invoke c-Fos expression in the superficial laminae of the dorsal horn.[38] Temperature preference test was performed to determine the temperature range of the cold hypersensitivity. Oxaliplatin-administered mice showed a greater preference to the plate with a fixed temperature of 24°C compared with control when the test plate was set at 15° and 10°C, whereas preference did not differ between groups when the test plate was set below 10°C. It has been suggested that temperature threshold for eliciting cold pain is less than 10°C and that temperature of 10°C or higher is innocuous.[42] Thus mice showed cold hypersensitivity at innocuous cold temperature. The temperature range also suggested that the cold hypersensitivity was likely mediated by transient receptor potential ankyrin 1 channels.[43,44] The results taken together, the cold hypersensitivity in mice observed in this study mimicked cold allodynia observed in patients. Patients may experience cold-induced symptoms as early as the time of drug infusion. We could not detect such early manifestation of cold hypersensitivity in naïve mice given a single dose of oxaliplatin. There are no

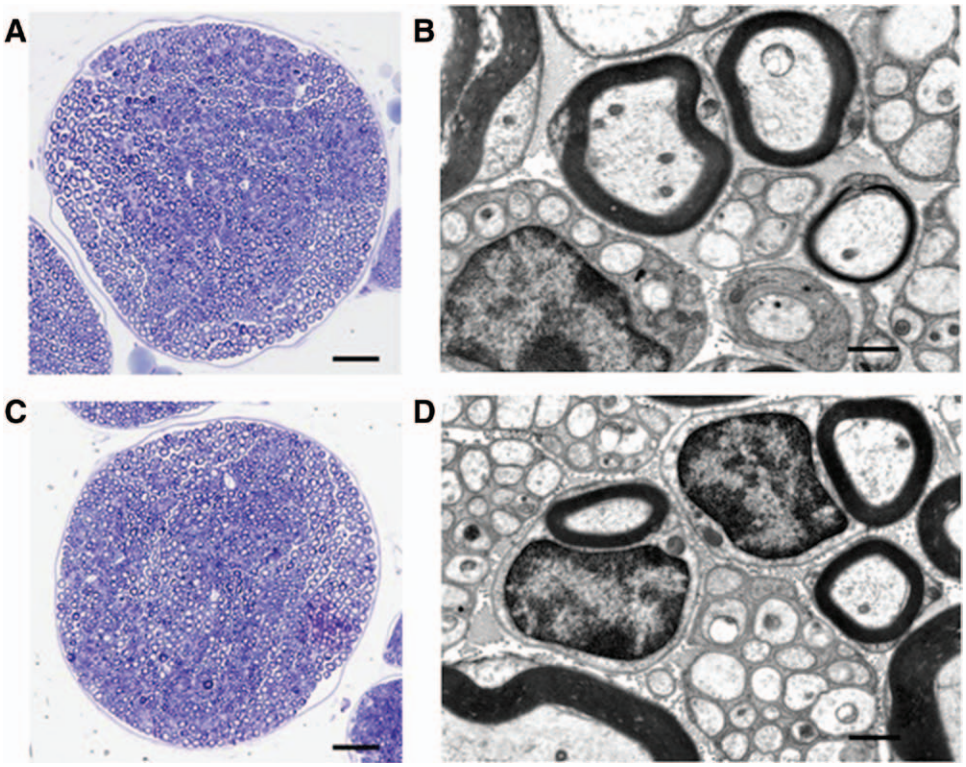

Fig. 4. Light and electron microscopic photographs of sciatic nerves harvested from a chronic vehicle-administered mouse (*A* and *B*, respectively) and a chronic oxaliplatin-administered mouse (nine weekly administrations of 10 mg/kg doses) (*C* and *D*, respectively). The sciatic nerve specimens were harvested 4 days after the last administration. *Scale bars*, *A* and *C*: 50 μm; *B* and *D*: 1 μm.

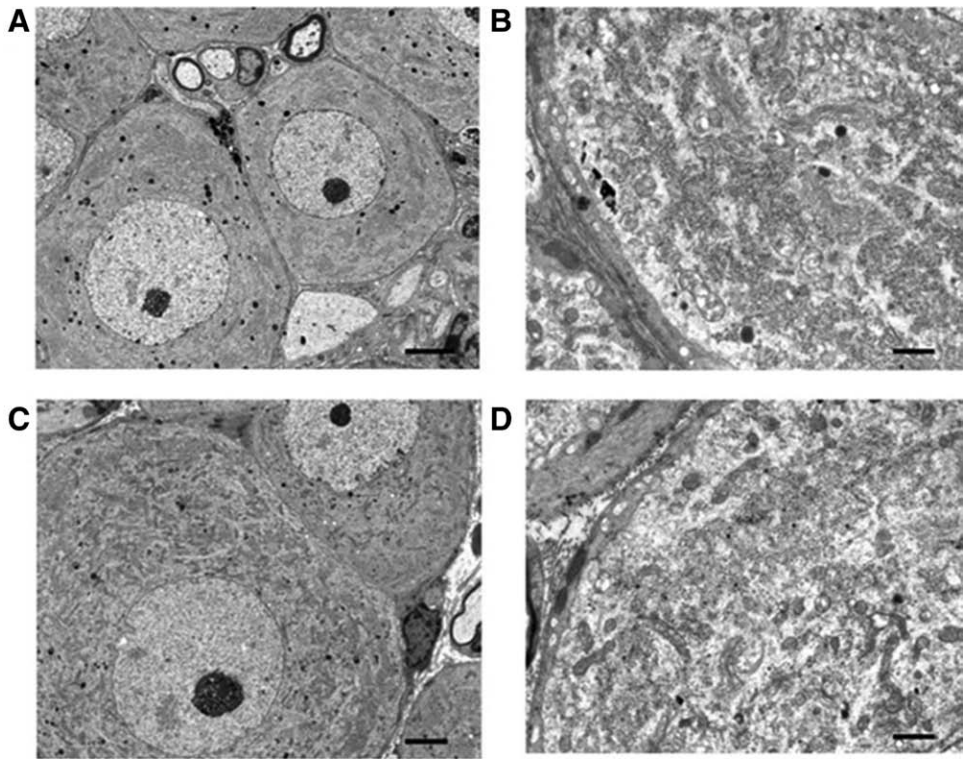

Fig. 5. Electron microscopic photograph of L4 dorsal root ganglion neurons of vehicle-administered mice (*A* and *B*) and chronic oxaliplatin-administered mice (nine weekly administrations of 10 mg/kg doses) (*C* and *D*). Specimens were harvested 4 days after the last administration. *Scale bars*, *A* and *C*: 5 μm; *B* and *D*: 1 μm.

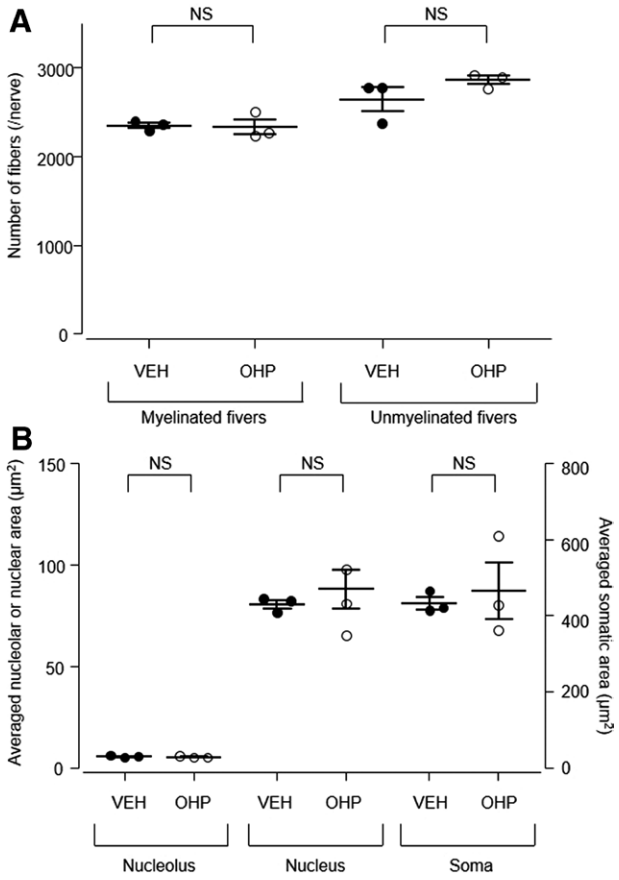

Fig. 6. Axon counts of sciatic nerves and morphometric analysis of dorsal root ganglion (DRG) neurons in vehicle-administered and chronic oxaliplatin-administered mice (three weekly administrations of 15 mg/kg doses) (n = 3 for each group). The sciatic nerve specimens and DRG neurons were harvested 4 days after the last administration. The number of myelinated and unmyelinated fibers of the sciatic nerve section taken just distal to the point where the posterior biceps semitendinosus nerve branches off was counted. The areas of nucleoli, nuclei, and somata of L4 DRG neurons were measured and the values were averaged for each mouse. Data were given as scatter plots of the number of myelinated and unmyelinated fibers (*A*), and nucleolar, nuclear, and somatic area of L4 DRG neurons (*B*) with mean ± SEM, and analyzed by two-tailed paired *t* test. NS = not significant; OHP = oxaliplatin; VEH = vehicle.

studies in patients examining the actual time course of cold hypersensitivity, that is, peak time and duration, thus we cannot ascertain that the time course observed in our study is comparable with patients. Furthermore, repeated administrations might cause change in time course such as early onset of the symptom. It is not clear whether patients with early onset of cold hypersensitivity were having their first injection or had previous administrations.

Chronic neuropathy develops in patients after repetitive oxaliplatin dosing, and once developed, most patients experience symptoms for months.[5] Patients complain of ongoing dysesthesia/paresthesia of hands and feet, which are frequently accompanied by impaired sensorymotor coordination of extremities.[3,7,8] Evaluating possible ongoing dysesthesia/paresthesia in animals may be challenging.[45] We did not detect any increase in c-Fos expression in the spinal dorsal horn in mice that were given repeated oxaliplatin treatment. Similarly, no increase in c-Fos expression was induced in a neuropathic pain model induced by chronic constriction of sciatic nerves in rats.[46] Repeated oxaliplatin administrations gradually induced a decrease in mechanical threshold, indicative of mechanical allodynia, which persisted for weeks after cessation of treatment. We cannot affirm that this mechanical allodynia observed in mice represents chronic neuropathy in patients. However, other investigators have demonstrated that after repeated oxaliplatin administration, animals developed mechanical hypersensitivity as well as reduction in conduction velocity of sensory nerves,[12,17,18] latter of which is also observed in patients with oxaliplatin-induced chronic neuropathy.[47–49] Attal *et al.*[3] reported that they could not detect any decrease in mechanical pain threshold in patients, whereas some patients complained of brush- or pressure-evoked pain.[4,6] On the basis of our personal clinical experience, we have observed that although patients with oxaliplatin-induced ongoing dysesthesia/paresthesia feel numbness in their hands, they complain of increased dysesthesia when their feet are lightly touched. This may be the symptom that is manifested as mechanical allodynia in mice. Furthermore, the gradually developing and persistent nature of the mechanical allodynia in mice was similar to that of the symptoms of oxaliplatin-induced chronic neuropathy in patients. Thus, it is plausible to say that the mechanical allodynia observed in this study is a manifestation of oxaliplatin-induced chronic neuropathy and may be used as an indicator of the severity of chronic neuropathy in mice. An interesting finding of our study was that three weekly administrations of 15 mg/kg doses (cumulative dose: 45 mg/kg) induced mechanical allodynia, whereas nine weekly administrations of 5 mg/kg doses (cumulative dose: 45 mg/kg) did not. Although it is generally accepted clinically that the cumulative dose of oxaliplatin is the major factor that determines the occurrence of chronic neuropathy, our results show that the dose of each injection is also an important factor. This is consistent with current recommendation for management of oxaliplatin-induced neurotoxicity, that is, decrease dosage and increase number of administrations to reach the same cumulative dose.[8]

Importantly, our study is the first to show that, in mice as in patients, a single dose of oxaliplatin induced acute cold hypersensitivity that diminished during the rest period of the administration cycle and that repeating these administration cycles induced chronic neuropathy. The doses of oxaliplatin used in our study were greater than those used in previous studies. However, based on our results and studies on antitumor effects of oxaliplatin in mice,[19–21] the doses used in our study are appropriate because doses that produce

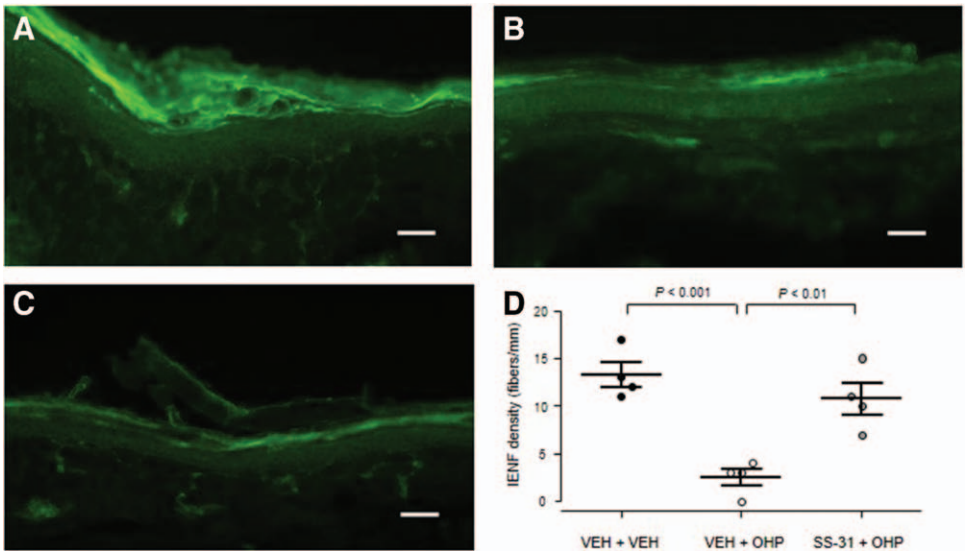

Fig. 7. Change in intraepidermal nerve fiber (IENF) density by chronic oxaliplatin administration with or without concomitant SS-31 administration. Fluorescence microscopic photographs of IENFs in plantar skin harvested from a mouse that received repeated injection of distilled water with continuous administration of saline (VEH + VEH) (*A*), a chronic oxaliplatin-administered mouse (three weekly administrations of 15 mg/kg doses) with continuous administration of saline (VEH + OHP) (*B*), and a chronic oxaliplatin-administered mouse (three weekly administrations of 15 mg/kg doses) with continuous administration of SS-31 (5 mg kg^{-1} day^{-1}) (SS-31 + OHP) (*C*). (*D*) Counts of IENFs per 1 mm of epidermal border. The skin specimens were harvested 4 days after the last administration of oxaliplatin or vehicle. The number of IENFs was counted in three random sections and averaged for each mouse (n = 4 for each group). *Scale bars* are 50 μm. Data were given as scatter plots of the number of IENFs with mean ± SEM and analyzed by one-way ANOVA ($P < 0.001$) followed by Bonferroni comparison tests (*D*). OHP = oxaliplatin; VEH = vehicle.

antitumor effects should be used to investigate side effects. Thus our model mimics conditions of patients with oxaliplatin-induced neuropathy and may be useful to further investigate the neuropathy and to reduce variability in results as observed in previous studies that used variable protocols for administration.[17,18,22,23,50–57]

In this study, we detected no overt morphological changes in peripheral nerves and DRGs even after nine weekly oxaliplatin administrations that induced severe mechanical allodynia. Thus we could not confirm previous reports that demonstrated morphological changes.[12–17] We observed vacuolation of some mitochondria in peripheral nerve axons of both control and oxaliplatin-treated mice. Vacuolation may be physiological[25] or may be an artifact produced during processing. Although the use of rats and different strains of mice might have contributed to the different results in other studies, oxaliplatin administration modality as discussed above may likely have caused the difference. It has been shown that peripheral neurotoxicity of oxaliplatin depended not only on the cumulative dose but also on the recovery time allowed between the doses.[7,12] Recently, no axonal degeneration in peripheral nerves was demonstrated in rats with oxaliplatin-induced neuropathy that presented slowed conduction and abnormal spontaneous discharge in sensory nerves and mechanical hypersensitivity.[18] Our results and previous reports taken together, it is suggested that degeneration of peripheral nerve axons is not necessary for the development of chronic neuropathic symptoms although axonal degeneration may result by chronic oxaliplatin treatment. However, IENF loss, previously reported in chemotherapy-induced neuropathy models,[18,57] was also present in our model and may be involved in the generation of neuropathic symptoms.

Recently, mitochondrial dysfunction and oxidative stress have been suggested to be involved in chemotherapy-induced neuropathies.[18,22–25,58,59] Oxaliplatin and paclitaxel cause functional impairment of peripheral nerve mitochondria which leads to electron leak and increased production of ROS.[18,24,25] SS-31 is a mitochondria-targeted antioxidant that has been shown to be neuroprotective in a variety of disease models that involve mitochondria dysfunction.[28–34] Continuous administration of SS-31 throughout repeated administration cycles of oxaliplatin reduced both cold hypersensitivity and mechanical allodynia and also mitigated IENF loss. Furthermore, we found that acute administration of SS-31 after the onset of symptoms could only reverse cold hypersensitivity but not mechanical allodynia. Our results indicate that SS-31 can protect against the development of chronic neuropathy but cannot affect its symptom once developed. The protective effects of SS-31 are most likely *via* reduction of mitochondrial ROS. We showed that repeated oxaliplatin administration resulted in increased levels of ROS/RNS in DRGs and that concomitant administration of SS-31

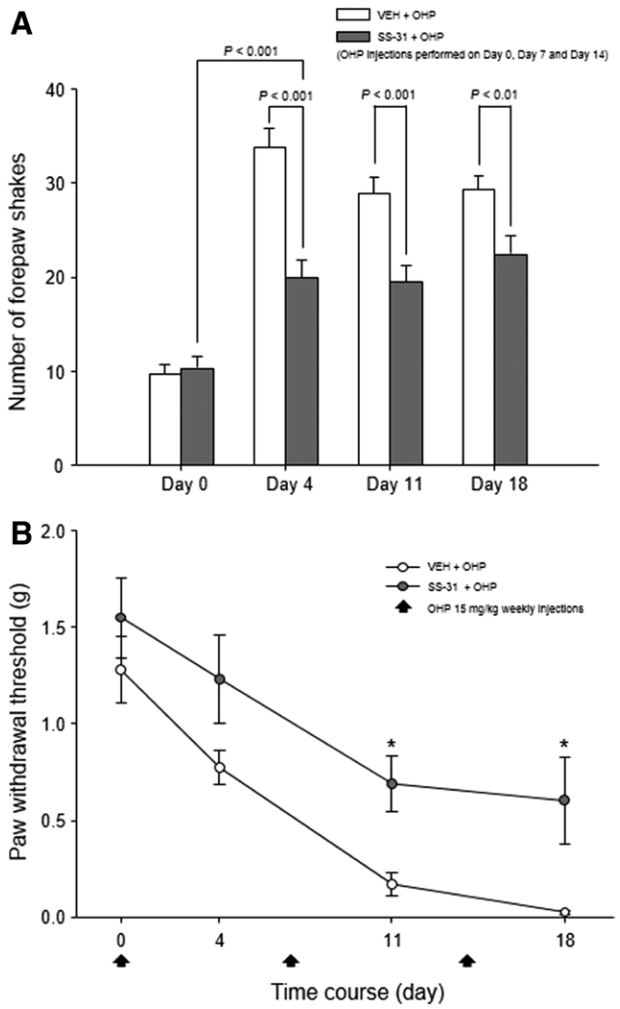

Fig. 8. Effects of continuous administration of SS-31 during chronic oxaliplatin administration on cold hypersensitivity and mechanical allodynia. SS-31 at 5 mg kg^{-1} day^{-1} or vehicle was continuously administered while mice were given three weekly administrations of oxaliplatin (each dose at 15 mg/kg). Tests were performed 4 days after each oxaliplatin injection to assess cold hypersensitivity and mechanical allodynia. (*A*) Cold hypersensitivity was assessed by the 15°C-cold plate test (see text) (n = 6 for each group). (*B*) Mechanical allodynia was assessed by the von Frey hair test (n = 6 for each group). Data were given as the mean ± SEM and analyzed by two-way ANOVA for repeated measures (*A*, group effect: $P < 0.001$; time effect: $P < 0.001$; *B*, group effect: $P = 0.029$; time effect: $P < 0.001$) followed by Bonferroni multiple comparison tests. * $P < 0.05$ *vs.* corresponding time point of control group (*B*). OHP = oxaliplatin; VEH = vehicle.

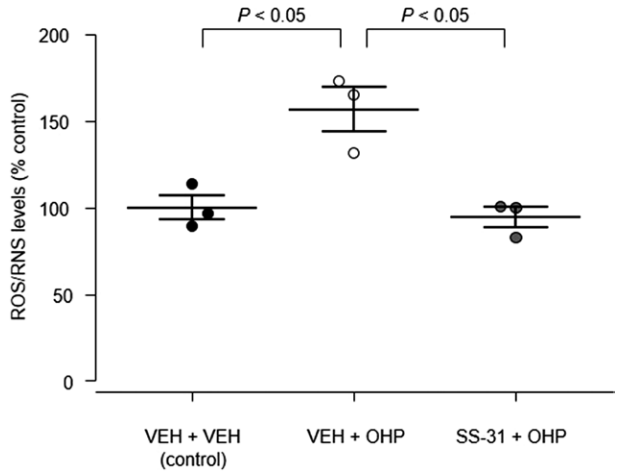

Fig. 9. Change in reactive oxygen species (ROS)/reactive nitrogen species (RNS) levels of lumbar dorsal root ganglion by chronic oxaliplatin administration with or without concomitant SS-31 administration. [VEH + VEH]: mice that received repeated injection of distilled water with continuous administration of saline, [VEH + OHP]: mice that received chronic oxaliplatin administration (three weekly administrations of 15 mg/kg doses) with continuous administration of saline, [SS-31 + OHP]: mice that received chronic oxaliplatin administration (three weekly administrations of 15 mg/kg doses) with continuous administration of SS-31 (5 mg kg^{-1} day^{-1}). Analysis was performed 4 days after the last injection of oxaliplatin or vehicle. Data were normalized to one dorsal root ganglion level and were expressed as percentage of the mean value of the control group (n = 3 for each group). Data were given as scatter plots of the ROS/RNS levels with mean ± SEM and analyzed by one-way ANOVA ($P = 0.021$) followed by Bonferroni comparison tests. OHP = oxaliplatin; VEH = vehicle.

attenuated the increase. Chronic neuropathy may involve chronic generation of mitochondrial ROS which induces neural changes that cannot be readily reversed by transient reduction of mitochondrial ROS. In contrast, SS-31 was able to alleviate cold hypersensitivity that was already present, indicating that it affected the maintenance of cold hypersensitivity. Increase in c-Fos expression after cold stimulation in mice with cold hypersensitivity was also attenuated by SS-31. The acute neuropathy may be caused directly by mitochondrial ROS, for example, mitochondrial ROS may directly affect transient receptor potential channels[50,54,60–63] and/or voltage-gated sodium and potassium channels.[50,64–68]

In summary, this murine model of oxaliplatin-induced neuropathy mimics patients' conditions and will be useful to further investigate mechanisms underlying the neuropathy and to develop drugs for its prevention and treatment. Our results suggest that mitochondrial ROS is involved differently in the acute and chronic neuropathies, and that SS-31 may be a promising candidate for prevention of oxaliplatin-induced neuropathy.

Acknowledgments

The authors thank Soichiro Murata, M.D., Ph.D. (Tsukuba University Graduate School of Medicine, Tsukuba, Ibaraki, Japan), for providing us with HCT116 cell line and for the technical support for the experiment on antitumor effects of oxaliplatin.

The work was supported by a grant-in-aid for Scientific Research (grant no. 24592358) from the Japan Society for

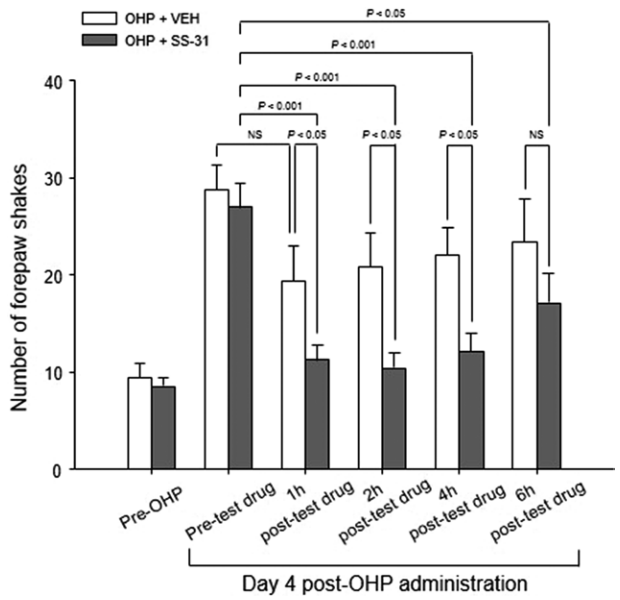

Fig. 10. Effects of acute administration of SS-31 on established oxaliplatin-induced neuropathic symptoms. Effect on cold hypersensitivity was examined in mice given a single dose of oxaliplatin (15 mg/kg) and tested 4 days after the injection. The 15°C-cold plate test was performed before and 1, 2, 4, and 6 h after subcutaneous administration of SS-31 (10 mg/kg) (n = 7) or vehicle (n = 5). Data were given as the mean ± SEM and analyzed by two-way ANOVA (group effect: $P = 0.021$; time effect: $P < 0.001$) followed by Bonferroni multiple comparison tests. NS = not significant; OHP = oxaliplatin; VEH = vehicle.

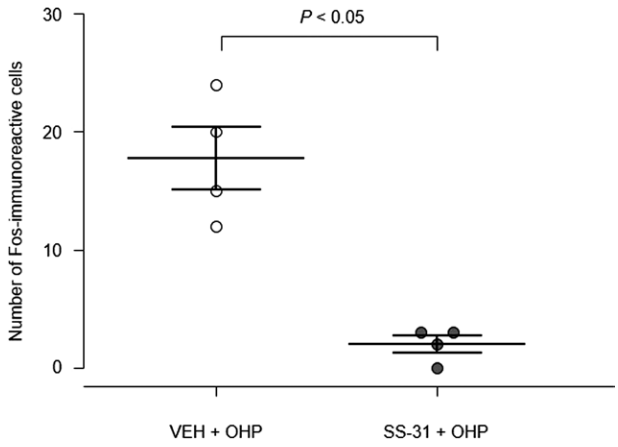

Fig. 11. Counts of Fos-like immunoreactive cells in the superficial laminae of the spinal dorsal horn (C7-T1 and L4-5 segments) after 15°C-stimulation of paws of oxaliplatin-administered mice with acute administration of SS-31 or vehicle. The experiment was performed 4 days after a single dose of oxaliplatin (15 mg/kg) or vehicle administration. Thermal stimulations were applied to the paws 2 h after the administration of SS-31 or vehicle. Ten repetitive stimulations lasting 30 s each were applied to the right forepaw and hind paw in 2-min cycles over a total period of 20 min, and mice were sacrificed after 2 h. The number of Fos-like immunoreactive nuclei in the superficial laminae of the ipsilateral dorsal horn in three random sections per segment was counted and averaged (n = 4 for each group). Data were given as scatter plots of the number of the Fos-like immunoreactive cells in vehicle- and oxaliplatin-administered mice with mean ± SEM and analyzed by two-tailed paired *t* test. OHP = oxaliplatin; VEH = vehicle.

the Promotion of Science (Tokyo, Japan), and by departmental sources of Teikyo University (Tokyo, Japan).

Competing Interests

Dr. Szeto has financial interest in the subject matter and materials discussed in this article. Dr. Szeto is the inventor of the SS peptides, including SS-31, described in this article. The Cornell Research Foundation (Ithaca, New York), holds patents on this technology, and Dr. Szeto is the inventor listed on the patents (USPTO no. 7550439, USPTO no. 7576061). Dr. Szeto is the Scientific Founder of Stealth Peptides International (Newton Centre, Massachusetts), and Cornell Research Foundation has licensed the technology to Stealth Peptides International for commercial development.

Correspondence

Address correspondence to Dr. Shimoyama: Department of Anesthesiology, Teikyo University Chiba Medical Center, 3426-3 Anesaki, Ichihara-shi, Chiba-ken 299-0111, Japan. mshimoy@med.teikyo-u.ac.jp. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

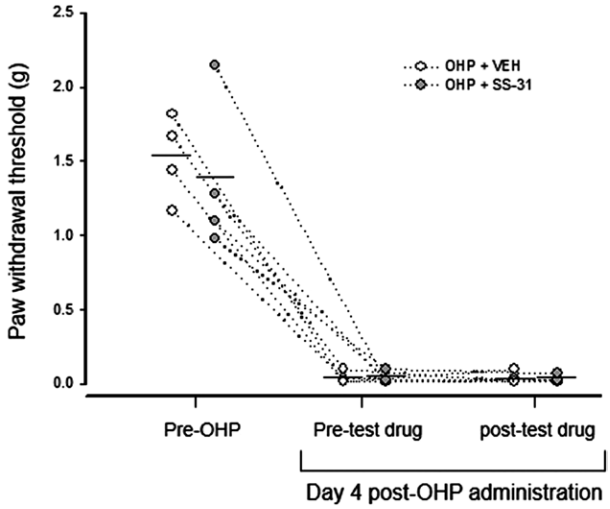

Fig. 12. Effect on mechanical allodynia was examined in mice given three weekly injections of oxaliplatin (each dose at 15 mg/kg) and tested 4 days after the third injection. Mechanical thresholds were measured by the von Frey hair test before and 2 h after the subcutaneous administration of SS-31 (10 mg/kg) or vehicle (n = 4 for each group). The *solid bar* represents the mean. Data were analyzed by two-way ANOVA (group effect: $P = 0.603$; time effect: $P < 0.001$). OHP = oxaliplatin; VEH = vehicle.

References

1. André T, Boni C, Mounedji-Boudiaf L, Navarro M, Tabernero J, Hickish T, Topham C, Zaninelli M, Clingan P, Bridgewater J, Tabah-Fisch I, de Gramont A; Multicenter International Study of Oxaliplatin/5-Fluorouracil/Leucovorin in the Adjuvant Treatment of Colon Cancer (MOSAIC) Investigators: Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer. N Engl J Med 2004; 350: 2343–51
2. Baker DE: Oxaliplatin: A new drug for the treatment of metastatic carcinoma of the colon or rectum. Rev Gastroenterol Disord 2003; 3:31–8
3. Attal N, Bouhassira D, Gautron M, Vaillant JN, Mitry E, Lepère C, Rougier P, Guirimand F: Thermal hyperalgesia as a marker of oxaliplatin neurotoxicity: A prospective quantified sensory assessment study. Pain 2009; 144:245–52
4. Binder A, Stengel M, Maag R, Wasner G, Schoch R, Moosig F, Schommer B, Baron R: Pain in oxaliplatin-induced neuropathy—Sensitisation in the peripheral and central nociceptive system. Eur J Cancer 2007; 43:2658–63
5. de Gramont A: Rapid evolution in colorectal cancer: Therapy now and over the next five years. Oncologist 2005; 10(suppl 2):4–8
6. Leonard GD, Wright MA, Quinn MG, Fioravanti S, Harold N, Schuler B, Thomas RR, Grem JL: Survey of oxaliplatin-associated neurotoxicity using an interview-based questionnaire in patients with metastatic colorectal cancer. BMC Cancer 2005; 5:116
7. Pasetto LM, D'Andrea MR, Rossi E, Monfardini S: Oxaliplatin-related neurotoxicity: How and why? Crit Rev Oncol Hematol 2006; 59:159–68
8. Saif MW, Reardon J: Management of oxaliplatin-induced peripheral neuropathy. Ther Clin Risk Manag 2005; 1:249–58
9. Wilson RH, Lehky T, Thomas RR, Quinn MG, Floeter MG, Grem JL: Acute oxaliplatin-induced peripheral nerve excitability. J Clin Oncol 2002; 20:1767–74
10. Raymond E, Chaney SG, Taamma A, Cvitkovic E: Oxaliplatin: A review of preclinical and clinical studies. Ann Oncol 1998; 9:1053–71
11. Extra JM, Espie M, Calvo F, Ferme C, Mignot L, Marty M: Phase I study of oxaliplatin in patients with advanced cancer. Cancer Chemother Pharmacol 1990; 25:299–303
12. Cavaletti G, Tredici G, Petruccioli MG, Dondè E, Tredici P, Marmiroli P, Minoia C, Ronchi A, Bayssas M, Etienne GG: Effects of different schedules of oxaliplatin treatment on the peripheral nervous system of the rat. Eur J Cancer 2001; 37:2457–63
13. Donzelli E, Carfì M, Miloso M, Strada A, Galbiati S, Bayssas M, Griffon-Etienne G, Cavaletti G, Petruccioli MG, Tredici G: Neurotoxicity of platinum compounds: Comparison of the effects of cisplatin and oxaliplatin on the human neuroblastoma cell line SH-SY5Y. J Neurooncol 2004; 67:65–73
14. McKeage MJ, Hsu T, Screnci D, Haddad G, Baguley BC: Nucleolar damage correlates with neurotoxicity induced by different platinum drugs. Br J Cancer 2001; 85:1219–25
15. Ta LE, Espeset L, Podratz J, Windebank AJ: Neurotoxicity of oxaliplatin and cisplatin for dorsal root ganglion neurons correlates with platinum-DNA binding. Neurotoxicology 2006; 27:992–1002
16. Jamieson SM, Liu J, Connor B, McKeage MJ: Oxaliplatin causes selective atrophy of a subpopulation of dorsal root ganglion neurons without inducing cell loss. Cancer Chemother Pharmacol 2005; 56:391–9
17. Renn CL, Carozzi VA, Rhee P, Gallop D, Dorsey SG, Cavaletti G: Multimodal assessment of painful peripheral neuropathy induced by chronic oxaliplatin-based chemotherapy in mice. Mol Pain 2011; 7:29
18. Xiao WH, Zheng H, Bennett GJ: Characterization of oxaliplatin-induced chronic painful peripheral neuropathy in the rat and comparison with the neuropathy induced by paclitaxel. Neuroscience 2012; 203:194–206
19. Banerjee S, Kong D, Azmi AS, Wang Z, Ahmad A, Sethi S, Sarkar FH: Restoring sensitivity to oxaliplatin by a novel approach in gemcitabine-resistant pancreatic cancer cells *in vitro* and *in vivo*. Int J Cancer 2011; 128:1240–50
20. Cividalli A, Ceciarelli F, Livdi E, Altavista P, Cruciani G, Marchetti P, Danesi DT: Radiosensitization by oxaliplatin in a mouse adenocarcinoma: Influence of treatment schedule. Int J Radiat Oncol Biol Phys 2002; 52:1092–8
21. Xiong W, Ren ZG, Qiu SJ, Sun HC, Wang L, Liu BB, Li QS, Zhang W, Zhu XD, Liu L, Wang WQ, Tang ZY: Residual hepatocellular carcinoma after oxaliplatin treatment has increased metastatic potential in a nude mouse model and is attenuated by Songyou Yin. BMC Cancer 2010; 10:219
22. Di Cesare Mannelli L, Zanardelli M, Failli P, Ghelardini C: Oxaliplatin-induced neuropathy: Oxidative stress as pathological mechanism. Protective effect of silibinin. J Pain 2012; 13:276–84
23. Joseph EK, Chen X, Bogen O, Levine JD: Oxaliplatin acts on IB4-positive nociceptors to induce an oxidative stress-dependent acute painful peripheral neuropathy. J Pain 2008; 9:463–72
24. Xiao WH, Bennett GJ: Effects of mitochondrial poisons on the neuropathic pain produced by the chemotherapeutic agents, paclitaxel and oxaliplatin. Pain 2012; 153:704–9
25. Zheng H, Xiao WH, Bennett GJ: Functional deficits in peripheral nerve mitochondria in rats with paclitaxel- and oxaliplatin-evoked painful peripheral neuropathy. Exp Neurol 2011; 232:154–61
26. Zhao K, Luo G, Giannelli S, Szeto HH: Mitochondria-targeted peptide prevents mitochondrial depolarization and apoptosis induced by tert-butyl hydroperoxide in neuronal cell lines. Biochem Pharmacol 2005; 70:1796–806
27. Zhao K, Zhao GM, Wu D, Soong Y, Birk AV, Schiller PW, Szeto HH: Cell-permeable peptide antioxidants targeted to inner mitochondrial membrane inhibit mitochondrial swelling, oxidative cell death, and reperfusion injury. J Biol Chem 2004; 279:34682–90
28. Calkins MJ, Manczak M, Mao P, Shirendeb U, Reddy PH: Impaired mitochondrial biogenesis, defective axonal transport of mitochondria, abnormal mitochondrial dynamics and synaptic degeneration in a mouse model of Alzheimer's disease. Hum Mol Genet 2011; 20:4515–29
29. Calkins MJ, Manczak M, Reddy PH: Mitochondria-targeted antioxidant SS31 prevents amyloid beta-induced mitochondrial abnormalities and synaptic degeneration in Alzheimer's disease. Pharmaceuticals (Basel) 2012; 5:1103–19
30. Cho S, Szeto HH, Kim E, Kim H, Tolhurst AT, Pinto JT: A novel cell-permeable antioxidant peptide, SS31, attenuates ischemic brain injury by down-regulating CD36. J Biol Chem 2007; 282:4634–42
31. Manczak M, Mao P, Calkins MJ, Cornea A, Reddy AP, Murphy MP, Szeto HH, Park B, Reddy PH: Mitochondria-targeted antioxidants protect against amyloid-beta toxicity in Alzheimer's disease neurons. J Alzheimers Dis 2010; 20(suppl 2):S609–31
32. Petri S, Kiaei M, Damiano M, Hiller A, Wille E, Manfredi G, Calingasan NY, Szeto HH, Beal MF: Cell-permeable peptide antioxidants as a novel therapeutic approach in a mouse model of amyotrophic lateral sclerosis. J Neurochem 2006; 98:1141–8
33. Reddy TP, Manczak M, Calkins MJ, Mao P, Reddy AP, Shirendeb U, Park B, Reddy PH: Toxicity of neurons treated with herbicides and neuroprotection by mitochondria-targeted antioxidant SS31. Int J Environ Res Public Health 2011; 8:203–21
34. Yang L, Zhao K, Calingasan NY, Luo G, Szeto HH, Beal MF: Mitochondria targeted peptides protect against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine neurotoxicity. Antioxid Redox Signal 2009; 11:2095–104

35. Zimmermann M: Ethical guidelines for investigations of experimental pain in conscious animals. Pain 1983; 16:109–10
36. Walczak JS, Beaulieu P: Comparison of three models of neuropathic pain in mice using a new method to assess cold allodynia: The double plate technique. Neurosci Lett 2006; 399:240–4
37. Beecher HK: The measurement of pain; prototype for the quantitative study of subjective responses. Pharmacol Rev 1957; 9:59–209
38. Knowlton WM, Bifolck-Fisher A, Bautista DM, McKemy DD: TRPM8, but not TRPA1, is required for neural and behavioral responses to acute noxious cold temperatures and cold-mimetics *in vivo*. Pain 2010; 150:340–50
39. Coggeshall RE: Fos, nociception and the dorsal horn. Prog Neurobiol 2005; 77:299–352
40. Shimoyama M, Tatsuoka H, Ohtori S, Tanaka K, Shimoyama N: Change of dorsal horn neurochemistry in a mouse model of neuropathic cancer pain. Pain 2005; 114:221–30
41. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL: Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods 1994; 53:55–63
42. Allchorne AJ, Broom DC, Woolf CJ: Detection of cold pain, cold allodynia and cold hyperalgesia in freely behaving rats. Mol Pain 2005; 1:36
43. Jordt SE, Bautista DM, Chuang HH, McKemy DD, Zygmunt PM, Högestätt ED, Meng ID, Julius D: Mustard oils and cannabinoids excite sensory nerve fibres through the TRP channel ANKTM1. Nature 2004; 427:260–5
44. Story GM, Peier AM, Reeve AJ, Eid SR, Mosbacher J, Hricik TR, Earley TJ, Hergarden AC, Andersson DA, Hwang SW, McIntyre P, Jegla T, Bevan S, Patapoutian A: ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. Cell 2003; 112:819–29
45. Mogil JS, Crager SE: What should we be measuring in behavioral studies of chronic pain in animals? Pain 2004; 112:12–5
46. Catheline G, Le Guen S, Honoré P, Besson JM: Are there long-term changes in the basal or evoked Fos expression in the dorsal horn of the spinal cord of the mononeuropathic rat? Pain 1999; 80:347–57
47. Burakgazi AZ, Messersmith W, Vaidya D, Hauer P, Hoke A, Polydefkis M: Longitudinal assessment of oxaliplatin-induced neuropathy. Neurology 2011; 77:980–6
48. Krishnan AV, Goldstein D, Friedlander M, Kiernan MC: Oxaliplatin-induced neurotoxicity and the development of neuropathy. Muscle Nerve 2005; 32:51–60
49. Pietrangeli A, Leandri M, Terzoli E, Jandolo B, Garufi C: Persistence of high-dose oxaliplatin-induced neuropathy at long-term follow-up. Eur Neurol 2006; 56:13–6
50. Descoeur J, Pereira V, Pizzoccaro A, Francois A, Ling B, Maffre V, Couette B, Busserolles J, Courteix C, Noel J, Lazdunski M, Eschalier A, Authier N, Bourinet E: Oxaliplatin-induced cold hypersensitivity is due to remodelling of ion channel expression in nociceptors. EMBO Mol Med 2011; 3:266–78
51. Joseph EK, Levine JD: Comparison of oxaliplatin- and cisplatin-induced painful peripheral neuropathy in the rat. J Pain 2009; 10:534–41
52. Ling B, Authier N, Balayssac D, Eschalier A, Coudore F: Behavioral and pharmacological description of oxaliplatin-induced painful neuropathy in rat. Pain 2007; 128:225–34
53. Ling B, Coudoré-Civiale MA, Balayssac D, Eschalier A, Coudoré F, Authier N: Behavioral and immunohistological assessment of painful neuropathy induced by a single oxaliplatin injection in the rat. Toxicology 2007; 234:176–84
54. Nassini R, Gees M, Harrison S, De Siena G, Materazzi S, Moretto N, Failli P, Preti D, Marchetti N, Cavazzini A, Mancini F, Pedretti P, Nilius B, Patacchini R, Geppetti P: Oxaliplatin elicits mechanical and cold allodynia in rodents *via* TRPA1 receptor stimulation. Pain 2011; 152:1621–31
55. Sakurai M, Egashira N, Kawashiri T, Yano T, Ikesue H, Oishi R: Oxaliplatin-induced neuropathy in the rat: Involvement of oxalate in cold hyperalgesia but not mechanical allodynia. Pain 2009; 147:165–74
56. Ta LE, Low PA, Windebank AJ: Mice with cisplatin and oxaliplatin-induced painful neuropathy develop distinct early responses to thermal stimuli. Mol Pain 2009; 5:9
57. Boyette-Davis J, Dougherty PM: Protection against oxaliplatin-induced mechanical hyperalgesia and intraepidermal nerve fiber loss by minocycline. Exp Neurol 2011; 229:353–7
58. Flatters SJ, Bennett GJ: Studies of peripheral sensory nerves in paclitaxel-induced painful peripheral neuropathy: Evidence for mitochondrial dysfunction. Pain 2006; 122:245–57
59. Kim HK, Zhang YP, Gwak YS, Abdi S: Phenyl *N*-tert-butylnitrone, a free radical scavenger, reduces mechanical allodynia in chemotherapy-induced neuropathic pain in rats. Anesthesiology 2010; 112:432–9
60. Anand U, Otto WR, Anand P: Sensitization of capsaicin and icilin responses in oxaliplatin treated adult rat DRG neurons. Mol Pain 2010; 6:82
61. Gauchan P, Andoh T, Kato A, Kuraishi Y: Involvement of increased expression of transient receptor potential melastatin 8 in oxaliplatin-induced cold allodynia in mice. Neurosci Lett 2009; 458:93–5
62. Kawashiri T, Egashira N, Kurobe K, Tsutsumi K, Yamashita Y, Ushio S, Yano T, Oishi R: L type Ca^{2+} channel blockers prevent oxaliplatin-induced cold hyperalgesia and TRPM8 overexpression in rats. Mol Pain 2012; 8:7
63. Zhao M, Isami K, Nakamura S, Shirakawa H, Nakagawa T, Kaneko S: Acute cold hypersensitivity characteristically induced by oxaliplatin is caused by the enhanced responsiveness of TRPA1 in mice. Mol Pain 2012; 8:55
64. Webster RG, Brain KL, Wilson RH, Grem JL, Vincent A: Oxaliplatin induces hyperexcitability at motor and autonomic neuromuscular junctions through effects on voltage-gated sodium channels. Br J Pharmacol 2005; 146:1027–39
65. Adelsberger H, Quasthoff S, Grosskreutz J, Lepier A, Eckel F, Lersch C: The chemotherapeutic oxaliplatin alters voltage-gated Na^+ channel kinetics on rat sensory neurons. Eur J Pharmacol 2000; 406:25–32
66. Benoit E, Brienza S, Dubois JM: Oxaliplatin, an anticancer agent that affects both Na^+ and K^+ channels in frog peripheral myelinated axons. Gen Physiol Biophys 2006; 25:263–76
67. Grolleau F, Gamelin L, Boisdron-Celle M, Lapied B, Pelhate M, Gamelin E: A possible explanation for a neurotoxic effect of the anticancer agent oxaliplatin on neuronal voltage-gated sodium channels. J Neurophysiol 2001; 85:2293–7
68. Kagiava A, Tsingotjidou A, Emmanouilides C, Theophilidis G: The effects of oxaliplatin, an anticancer drug, on potassium channels of the peripheral myelinated nerve fibres of the adult rat. Neurotoxicology 2008; 29:1100–6