Title:

Etodolac, a cyclooxygenase-2 inhibitor, attenuates paclitaxel-induced peripheral neuropathy in a mouse model of mechanical allodynia

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Running Title

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Section

Behavioral pharmacology
Abbreviations:

COX: cyclooxygenase

DRG: dorsal root ganglion

NSAIDs: non-steroidal anti-inflammatory drugs

TRP channel: transient receptor potential channel

Chemical Structures of compounds

paclitaxel: \((2\alpha,4\alpha,5\beta,7\beta,10\beta,13\alpha)-4,10\text{-bis(acetyloxy)-13-\{[(2R,3S)-3-(benzoylamino)-2-hydroxy-3-phenylpropanoyl]oxy}\}-1,7\text{-dihydroxy-9-oxo-5,20-epoxytax-11-en-2-yl benzoate}\)

etodolac: \((RS)-2\text{-}(1,8\text{-Diethyl-4,9-dihydro-3H-pyrano\[3,4-b\]indol-1-yl)acetic acid}\)

indomethacin:

\(2\text{-}{1\text{-}\{[\text{4-chlorophenyl})carbonyl\}-5\text{-methoxy-2-methyl-1H-indol-3-yl\}acetic acid}\)

celecoxib: \(4\text{-}{5\text{-}(4\text{-methylphenyl)}-3\text{-}(trifluoromethyl)pyrazol-1-yl\}benzenesulfonamide}\)

diclofenac: \(2\text{-}(2\text{-}(2,6\text{-dichlorophenylamino)phenyl)acetic acid}\)

pregabalin: \((S)-3\text{-}(aminomethyl)-5\text{-methylhexanoic acid}\)

duloxetine: \((+)-(S)-\text{N-Methyl-3-(naphthalen-1-yloxy)}\)-3\text{-}(thiophen-2-yl)propan-1-amine\)

mexiletine: \((RS)-1\text{-}(2,6\text{-dimethylphenoxy)propan-2-amine}\)
Abstract

The effect of the cyclooxygenase 2 (COX-2) inhibitor etodolac on the mechanical allodynia induced by paclitaxel was investigated in mice and compared with the effects of the nonselective COX inhibitors indomethacin and diclofenac, the selective COX-2 inhibitor celecoxib, the calcium channel α2δ subunit inhibitor pregabalin, the sodium channel blocker mexiletine, and the serotonin–norepinephrine reuptake inhibitor duloxetine. The decrease in the paw-withdrawal threshold induced by paclitaxel was reversed by oral administration of etodolac at 10 mg/kg, but was not affected by indomethacin, diclofenac or celecoxib. The antiallodynic effect of etodolac gradually increased during repeated administration, and after two weeks the paw-withdrawal threshold at the preadministration point was significantly increased. Pregabalin, duloxetine and mexiletine also showed an antiallodynic effect in this model. Whereas pregabalin had a preadministration effect similar to that of etodolac during repeated administration, mexiletine or duloxetine had no such effect. There was almost no difference in the distribution of etodolac and diclofenac in nervous tissue, indicating that COX inhibition is unlikely to be involved in the antiallodynic effect of etodolac.

Etodolac did not show a neuroprotective effect against morphological transformations such as the axonal degeneration induced by paclitaxel. Instead, etodolac probably acts at
the level of functional changes accompanying paclitaxel treatment, such as alterations in
the activation state of components of the pain-transmission pathway. Our findings
suggest that etodolac attenuates paclitaxel-induced peripheral neuropathy by a
COX-independent pathway and that it might be useful for the treatment of
paclitaxel-induced peripheral neuropathy.
Introduction

Paclitaxel is widely used to treat patients with cancers of the lung, breast, stomach, endometrium or ovary [Kohler DR and Goldspiel BR, 1994]. However, it causes a wide range of side effects such as vomiting, hematopoietic disorders and painful peripheral neuropathy [Rowinsky EK et al., 1993; Kohler DR and Goldspiel BR, 1994; Cata JP et al., 2006; Hausheer FH et al., 2006]. Paclitaxel-induced peripheral neuropathy becomes more marked with continued therapy, and it is often the dose-limiting toxicity. Although massage therapy, amino acids, vitamin supplements and analgesics have been used to treat this neuropathy, their efficacy is low and there is no preventive therapy [Vahdat L et al., 2001; Hausheer FH et al., 2006; Kaley TJ and DeAngelis LM, 2009].

Paclitaxel-treated mice or rats have been used as animal models of paclitaxel-induced peripheral neuropathy. These models reproduce many of the symptoms of paclitaxel-induced peripheral neuropathy in humans, including mechanical allodynia, thermal hyperalgesia, and thermal hypoalgesia [Polomano RC et al., 2001; Cata JP et al., 2006; Xiao W et al., 2008]. Various drugs have been investigated in these models, and gabapentin (a calcium channel α2δ subunit blocker) and mexiletine (a sodium channel blocker) were found to be effective in reducing the symptoms [Kamei J et al., 2006; Matsumoto M et al., 2006; Xiao W et al., 2007, 2008; Gauchan P et al.,...
Although prostanoids produced by cyclooxygenase 2 (COX-2) mediate the inflammatory cascade and have been suggested to contribute to the development of peripheral neuropathy in animal models [Ma W and Eisenach JC, 2002, 2003; Durrenberger PF et al., 2004; Schäfers M et al., 2004], COX inhibitors are not considered effective in the clinical management of neuropathy. However, we have shown that the COX-2 inhibitor etodolac strongly ameliorates the mechanical allodynia induced by partial sciatic nerve ligation in mice, whereas other COX inhibitors do not [Inoue N et al., 2009]. It is therefore possible that etodolac would also ameliorate paclitaxel-induced neuropathy.

In the present study, we investigated the effect of etodolac on mechanical allodynia in a mouse model and compared it with that of other COX inhibitors and analgesic agents used to treat peripheral neuropathic pain. Etodolac, but not other COX inhibitors, ameliorated the mechanical allodynia induced by paclitaxel.
Materials and methods

Experimental animals

This study was conducted in compliance with the Internal Regulations on Animal Experiments at Nippon Shinyaku Co., Ltd, which are based on the Law for the Humane Treatment and Management of Animals (Law No. 105, 1 October 1973, as amended on 1 June 2006). Male ddY mice aged five or six weeks were purchased from Japan SLC (Hamamatsu, Japan). They were housed four to six per cage under a 12-h light–dark cycle (lights on, 8:00–20:00) at 20–26°C, a relative humidity of 35–75%, and a ventilation frequency of at least 15 times/h. They were allowed free access to pellet chow (F-2; Funabashi Farm, Funabashi, Japan) and tap water.

Reagents

Paclitaxel was purchased from Xi’an Tiancheng Drugs & Bio-engineering (Xian, Shaanxi, China), diclofenac from Daiwa Pharmaceutical (Toyama, Japan), and indomethacin and mexiletine from Sigma-Aldrich (St. Louis, MO, USA). Etodolac, pregabalin and duloxetine were synthesized and celecoxib was extracted from Celebrex capsules (Pfizer, New York, NY, USA) in our laboratories. Etodolac, indomethacin, celecoxib, diclofenac, pregabalin, duloxetine, and mexiletine were suspended in 0.5%...
methylcellulose solution and administered orally through a stainless-steel gavage tube.

Mouse model of paclitaxel-induced allodynia

Paclitaxel was dissolved in ethanol:cremophor:saline (5:5:90, v/v/v) to a concentration of 0.4 mg/ml and injected intraperitoneally at a dose of 4 mg/kg on four nonconsecutive days (days 0, 2, 5, and 7) as previously described [Matusmoto M et al., 2006]. Mice injected with ethanol:cremophor:saline vehicle only were used as normal mice. For therapeutic treatment, drugs were administered orally once a day for two weeks from day 8, and mice injected with 0.5% methylcellulose vehicle only were used as control mice. The paw-withdrawal threshold (PWT) was measured with von Frey filaments (North Coast Medical, Morgan Hill, CA, USA) as described below once a week (days 8, 15, and 22) before drug administration and either 0.5 and 4 h or 1 and 4 h after administration.

Assessment of mechanical allodynia (von Frey filament test)

Mechanical allodynia was assessed as previously described [Inoue N et al., 2009]. Briefly, each mouse was acclimated alone in a perspex box on a raised metal mesh for at least 30 min before the test. von Frey filaments with bending forces of 0.008, 0.02, 0.04,
0.07, 0.16, 0.4, 0.6, 1.0, 1.4, 2, and 4 g were used. A filament was applied perpendicularly to the center of the plantar surface of the paw until the filament bent slightly. If the mouse withdrew or lifted the paw, a filament one size smaller was tried. Conversely, if no response was observed, a filament one size larger was tried. In this way, the minimum filament size (in grams) required to produce a positive response in at least three of 10 trials was determined and taken as the value of PWT.

**Cell proliferation assay**

The human non-small-cell lung cancer cell line A549 was purchased from Health Science Research Resources Bank (Osaka, Japan) and the human breast cancer cell line MDA-MB-468 and the human ovarian cancer cell line OVCAR were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO₂. For the proliferation assay, cells were plated onto 96-well plates at a density of 3000 (A549) or 6000 (MDA-MB-468 and OVCAR) cells/well. After preincubation for 16 h, paclitaxel and etodolac were added and the cells were incubated for a further 72 h at 37°C. Cell viability was then assessed by tetrazolium dye reduction. Briefly, the cells were incubated with 10 μL of TetraColor...
One cell proliferation assay reagent (Seikagaku, Tokyo, Japan) for 2 h at 37°C and the amount of formazan produced was determined by measuring the absorbance at 450 nm with a microplate reader (Bio-Rad, Hercules, CA, USA). Each treatment group was plated in triplicate, and the cell viability at each drug concentration was calculated as a percentage of that of untreated cells.

**Distribution of etodolac and diclofenac in central and peripheral nervous tissue**

\(^{14}\)C-Etodolac (100 \(\mu\)Ci/kg) was synthesized in our laboratories and \(^{14}\)C-diclofenac (25 \(\mu\)Ci/kg) was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA, USA). Mice were injected with paclitaxel (4 mg/kg) intraperitoneally on four alternate days (days 0, 2, 4, and 6). \(^{14}\)C-Etodolac (10 mg/kg) or \(^{14}\)C-diclofenac (3 mg/kg) was administered orally on day 7 (single administration) or once a day for seven days from day 7 (multiple administration). Four hours after the administration on day 7 or 14, blood samples were collected from the orbital sinus under isoflurane anesthesia, the mice were sacrificed by exsanguination, and the cerebrum, spinal cord, sciatic nerves and dorsal root ganglia (DRG) were removed. Plasma was prepared and tissue samples (<100 mg) were weighed and solubilized by the addition of 1 ml Solvable™ (PerkinElmer Life and Analytical Sciences) followed by heating at 40°C.
overnight. Plasma (0.1 ml) or solubilized tissue samples were mixed with 10 ml of scintillation fluid (Hionic-Fluor™ for tissues and Emulsifier Scintillator Plus™ for plasma; PerkinElmer Life and Analytical Sciences) and the radioactivity of the samples was measured with a liquid scintillation counter (Tri-Carb 3100TR; PerkinElmer Life and Analytical Sciences).

**Histopathological analysis**

Mice were injected with paclitaxel (20 mg/kg) intravenously on five nonconsecutive days (days 1, 3, 6, 8, and 10). Etodolac (10 mg/kg) was administered to paclitaxel-treated animals orally once a day for 17 days from day 0. All mice were euthanized by exsanguination under ether anesthesia on day 17 and the sciatic nerves and lumbar spinal cord with the dorsal root were removed. A portion of the sciatic nerves, as well as the lumbar spinal cord with the dorsal root, were fixed in 10% neutral-buffered formalin, embedded in paraffin, cut into 4-µm sections, and stained with hematoxylin and eosin for histopathological examination. The severity of the histopathological changes were classified as − (normal), ± (minimal), + (mild), ++ (moderate) or +++ (marked). The remaining sciatic nerves were fixed in 4% formaldehyde:glutaraldehyde:osmium tetroxide (98:1:1, v/v/v), embedded in epoxy
resin, cut into semi-thin (1-μm) cross-sections and stained with toluidine blue. The total area of the specimens was measured by image filing software (FLVFS-LS; Flovel, Tokyo, Japan), and the number of degenerating nerve fibers was counted under a microscope.

Statistical analysis

In the paclitaxel-induced allodynia model, statistically significant differences in PWT between normal groups (mice not treated with paclitaxel) and control groups (paclitaxel-treated mice not treated with test compounds) were determined by the Wilcoxon rank-sum test and between control and drug-treated groups by the Wilcoxon or Steel test. Statistically significant differences in the frequency of histopathological findings and the number of degenerating nerve fibers between normal and control groups and between control and etodolac-treated groups were determined by the Wilcoxon test. Values of $p < 0.05$ were regarded as statistically significant. Statistical analysis was carried out with the SAS system (version 8.2, SAS Institute, Cary, NC, USA).
Results

Effect of etodolac and other COX inhibitors on paclitaxel-induced mechanical allodynia in mice

Within one day of the last paclitaxel injection, the average paw-withdrawal threshold (PWT) of the control group had decreased from the pretreatment value of 0.41 g to 0.03 g (that of normal mice was 0.59 g; Fig. 1A). This pronounced mechanical allodynia lasted for at least two weeks after the end of paclitaxel treatment. Etodolac (10 mg/kg p.o.) increased the PWT observed from 0.5 h after the first administration. The efficacy gradually increased throughout the administration period, and the preadministration PWT was also increased on days 15 and 22. The antiallodynic effects of etodolac at 0.5 and 4 h after administration on day 15 and at preadministration, 0.5 and 4 h on day 22 were statistically significant. Indomethacin (1 mg/kg p.o.), celecoxib (30 mg/kg p.o.) and diclofenac (3 mg/kg p.o.) had no effect on PWT throughout the assessment period (Fig. 1 A and B).

Effect of pregabalin, duloxetine and mexiletine on paclitaxel-induced mechanical allodynia in mice

For comparison with etodolac, the antiallodynic effects of pregabalin (a calcium
channel α2δ subunit blocker), duloxetine (a serotonin–norepinephrine reuptake inhibitor), and mexiletine (a sodium channel blocker), were examined in the mouse model of mechanical allodynia. Pregabalin (30 mg/kg p.o.) significantly increased the PWT at 0.5 and 4 h after administration on days 8, 15, and 22 (Fig. 2A). Until day 15, the preadministration PWT was not significantly different from the control value, but it had significantly increased by day 22. Duloxetine at 30 and 100 mg/kg p.o. increased the PWT of paclitaxel-treated mice (Fig. 2B), but these doses tended to cause sedation. Mexiletine at 30 and 100 mg/kg p.o. similarly increased the PWT of paclitaxel-treated mice (Fig. 2C), but these doses also caused sedation.

**Effect of etodolac, pregabalin, duloxetine and mexiletine on the PWT of normal mice**

The effect on the PWT of normal mice of etodolac, pregabalin, duloxetine and mexiletine, which had antiallodynic effects in paclitaxel-treated mice, was examined (Fig. 3). Etodolac (10 mg/kg) did not affect the PWT of normal mice after a single or repeated administration. Duloxetine and mexiletine also had no effect on the PWT of normal mice after administration on day 0, but they slightly decreased the PWT at 1 and 4 h after administration on day 8. Pregabalin, however, increased the PWT of normal
mice at 1 and 4 h after administration on days 0 and 8, and the effect at 4 h on day 0 was significant.

**Effect of etodolac on antiproliferative activity of paclitaxel**

It is important that etodolac attenuates only the adverse effect of paclitaxel without weakening its antitumor activity. We therefore examined the antiproliferative effect of paclitaxel on human cancer cell lines in the presence of increasing concentrations of etodolac. Etodolac itself did not have an antiproliferative effect on the human lung adenocarcinoma epithelial cell line A549, and it also did not affect the antiproliferative activity of paclitaxel against that cell line (Fig. 4A). At 100 μM, the highest dose tested, etodolac slightly inhibited the proliferation of the human breast adenocarcinoma cell line MDA-MB468 (Fig. 4B) and the human ovarian carcinoma cell line OVCAR (Fig. 4C). In addition, etodolac tended to increase the antiproliferative activity of paclitaxel against MDA-MB468 cells (Fig. 4B).

**Nerve-tissue distribution of 14C-etodolac and 14C-diclofenac in normal and paclitaxel-treated mice**

Unlike other COX inhibitors, etodolac reduced paclitaxel-induced allodynia, and
we hypothesized that this finding might be explained by differences in the tissue distribution of etodolac and other COX inhibitors, especially in nervous tissues. We therefore compared the nerve-tissue distribution of etodolac with that of diclofenac in normal and paclitaxel-treated mice. The radioactivity levels in plasma, cerebrum, spinal cord, sciatic nerves and DRG (dorsal root ganglia) were measured after a single dose or seven doses of $^{14}$C-etodolac (10 mg/kg) or $^{14}$C-diclofenac (3 mg/kg) to normal and paclitaxel-treated mice (Fig. 5; Table 1). There was no appreciable difference in the distribution of etodolac and diclofenac when the ratios of the tissue concentration to the plasma concentration were compared for the cerebrum and spinal cord (central nervous tissue) and the sciatic nerve and DRG (peripheral nervous tissue) by single and multiple administrations in normal mice. By the same measure, there was little difference in the distribution of these compounds in the central and peripheral nervous systems in paclitaxel-treated mice (Fig. 5).

**Histopathology of peripheral nervous tissue in normal and paclitaxel-treated mice**

To assess whether the mechanism of action of etodolac might involve a neuroprotective effect, we performed histopathological examination of dorsal root, dorsal cord and sciatic nerves in normal and paclitaxel-treated mice. In a preliminary
experiment, paclitaxel at a dose of 20 mg/kg, but not 4 mg/kg, induced morphological injury such as vacuolization and axonal degeneration in peripheral nervous tissues. We therefore selected 20 mg/kg as a suitable dose of paclitaxel for morphological evaluation of nervous tissue. Paclitaxel induced minimal-to-mild vacuolization and axonal degeneration in all nervous tissues except dorsal cord with statistical significance when compared with nervous tissue from untreated animals, and etodolac had no noticeable neuroprotective effect (Fig. 6A–F; Table 2). Paclitaxel treatment also led to significantly increased numbers of degenerating nerve fibers, and etodolac treatment again had no noticeable histopathological improvement (Fig. 6G–I; Table 3).
Discussion

Paclitaxel-induced peripheral neuropathy occurs in 59–78% of patients receiving paclitaxel therapy, and its severity is proportional to the cumulative dose [Kohler DR and Goldspiel BR, 1994; Hausheer FH., 2006]. Peripheral neuropathy often becomes the dose-limiting toxicity for paclitaxel, and it often continues to affect patients even after the end of paclitaxel treatment. Although peripheral neuropathy is an important issue in clinical practice, there is no effective treatment for it, so that there is an urgent need for novel therapeutics.

The present study provides pharmacological evidence that the COX-2 inhibitor etodolac improves paclitaxel-induced peripheral neuropathy in a mouse model of mechanical allodynia. Etodolac attenuated paclitaxel-induced mechanical allodynia in this model, but the COX inhibitors indomethacin, diclofenac and celecoxib did not. The dosage of each COX inhibitor was chosen to be sufficient to produce antiinflammatory effects in our previous study [Inoue N et al., 2009] and in other studies [Inoue K et al., 1991; Katagiri M et al., 2006] in rodent models. The antiallodynic effect of etodolac was cumulative and after two weeks’ administration it was observed at the preadministration point. Because the half-life of etodolac administered at 10 mg/kg in mice has been reported to be only 13.2 h [Honda K et al., 1991], this delayed effect cannot be
attributed to residual drug. The effects of the analgesic agents pregabalin, mexiletine
and duloxetine, which are used to treat peripheral neuropathic pain, were tested in the
same model. The effect of pregabalin was similar to that of etodolac in that it gradually
increased its effect on repeated administration and showed an effect at the
preadministration point after two weeks’ administration. Mexiletine and duloxetine
showed no such effect. However, pregabalin increased the paw-withdrawal threshold in
normal mice, whereas etodolac did not. This result suggests that etodolac has
therapeutic efficacy against paclitaxel-induced peripheral neuropathy without
suppressing normal nerve activity.

The mechanism by which etodolac suppresses paclitaxel-induced peripheral
neuropathy is unknown. Before investigating the mechanism, we first tested whether
etodolac affected the antitumor activity of paclitaxel. Though etodolac did not affect the
antiproliferative activity of paclitaxel against A549 cells, it slightly increased the
antiproliferative activity of paclitaxel against MDA-MB-468 cells. In MDA-MB-468
and OVCAR cells, etodolac itself also inhibited cell proliferation at the highest dose
tested. Because the maximum drug concentration of etodolac administered at 10 mg/kg
in mice has been reported to be 33 μM [Honda K et al., 1991], the dosage of etodolac in
this examination corresponded approximately to the blood concentration of that used in
the allodynia model. These results provide evidence etodolac would not suppress the antitumor activity of paclitaxel. Furthermore, the slight antiproliferative effect of etodolac and its enhancement of the antiproliferative effect of paclitaxel in MDA-MB468 cells observed in the present study is consistent with other basic studies showing that etodolac has antineoplastic effects against a variety of cancers, including prostate [Kolluri SK et al., 2005; Khwaja FS et al., 2009], bladder [Okamoto A et al., 2008], skin [Kapadia GJ et al., 2010], and tongue carcinoma [Mishima K et al., 2005], hepatoma [Behari J et al., 2007], and pancreatic [Adachi T et al., 2008] and blood cancer [Yasui H et al., 2005; de Souza Thiago L et al., 2009], as well as antimetastatic effects [Iwata C et al., 2007; Benish M et al., 2008; Tachimori A et al., 2008], and a clinical study showing that etodolac can provide secondary prevention of gastric cancer [Yanaoka K et al., 2010]. Therefore, administration of etodolac with paclitaxel may not only be useful for reducing the adverse neurological effects of paclitaxel but may even enhance its antitumor activity.

As the second part of our study on the mechanism by which etodolac suppresses paclitaxel-induced peripheral neuropathy, we investigated the distribution of etodolac in central and peripheral nervous tissues, because differences in nerve-tissue distribution between etodolac and other COX inhibitors might shed light on the antiallodynic effect...
of etodolac. Although the accumulation of etodolac in the central nervous system is known to be low [Honda K et al., 1991], the extent of its accumulation in the peripheral nervous system is unknown. We therefore compared the bioaccumulation of $^{14}$C-labeled etodolac and diclofenac in normal and paclitaxel-treated mice. With or without paclitaxel treatment, the distribution of etodolac not only in central but also in peripheral nervous tissue was very similar to the distribution of diclofenac. If the antiallodynic effect of etodolac were mediated by COX inhibition, one would expect the effect to arise from a special distribution of etodolac in the peripheral nervous system that is different from that of diclofenac. The fact that there was no appreciable difference in the nerve-tissue distribution of the two compounds suggests that the effect of etodolac is mediated by a mechanism other than COX inhibition.

As the third and final part of our study, we carried out a histopathological examination of peripheral nervous tissues in normal and paclitaxel-treated mice. The pathogenesis of paclitaxel-induced peripheral neuropathy has not been fully elucidated, but it has been suggested that paclitaxel promotes the aggregation of intracellular microtubules and stabilizes them [Hausheer FH et al., 2006]. This would inhibit axonal transportation in neuronal cells, resulting in the disruption of neuronal function. In rodent models of paclitaxel-induced peripheral neuropathy, high doses of paclitaxel...
induce organic injury in nervous tissues, such as axonal degeneration and demyelination

[Authier N et al., 2000; Persohn E et al., 2005], and even low doses of paclitaxel induce changes in interneuron function such as altered expression of various channels, resulting in peripheral neuropathy [Alessandri-Haber N et al., 2004; Matsumoto M et al., 2006; Xiao W et al., 2007; Nishida K et al., 2008; Miyao K et al., 2009]. In the present study, etodolac had no effect on the axonal degeneration induced by paclitaxel in mice at the high dose of 20 mg/kg. Therefore etodolac did not have a direct neuroprotective action, and it probably acts by modulating the functional changes induced by paclitaxel.

Etodolac might, for example, modulate the gene expression or activation state of signal-transduction molecules such as the calcium channel α2δ subunit [Matusmoto M et al., 2006; Xiao W et al., 2007], which is the effectors site of pregabalin, matrix metalloprotease-3 [Nishida K et al., 2008], which has been suggested to activate macrophages, or members of transient receptor potential (TRP) channel families [Alessandri-Harber N et al., 2004; Miyano K et al., 2009], which are mechano- and thermo-sensitive receptors that transmit various stimuli, including pain. We are currently investigating the effects of etodolac on TRP channels.

In conclusion, etodolac ameliorated peripheral neuropathy in a paclitaxel-induced mouse model of alldynia. Etodolac appears to have analgesic effects that increase on
repeated treatment. The action of etodolac in paclitaxel-induced neuropathy is likely to be mediated by a mechanism other than COX inhibition, and the mechanism remains under investigation in our laboratory, with a focus on TRP channels. Etodolac has a good reputation for safety in long-term use because of the low level of gastrointestinal ulceration associated with its use, and it may contribute to the welfare of cancer patients suffering from the adverse neurological effects of paclitaxel chemotherapy.
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Authorship Contribution

Participated in research design: Ito, Nogawa, Inoue, Kyoji, Nakamura, Yamashita, Banno

Conducted experiments: Ito, Tajima, Nogawa, Inoue, Takahashi, Sasagawa, Kotera, Ueda

Contributed new reagents or analytic tools: None

Performed data analysis: Ito, Tajima, Nogawa, Sasagawa, Kotera

Wrote or contributed to the writing of the manuscript: Ito, Nogawa, Inoue
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Legends for Figures

Fig. 1. Effect of COX inhibitors on paclitaxel-induced mechanical allodynia in mice.
Etodolac, indomethacin and celecoxib (A) and diclofenac (B). Each symbol represents the mean PWT for 10 mice (A) or four mice (B). Drugs were administered orally once a day for two weeks from day 8. ## $p < 0.01$ (vs. Normal, Wilcoxon test), * $p < 0.05$, ** $p < 0.01$ (vs. Control, Steel test [A], Wilcoxon test [B])

Fig. 2. Effect of pregabalin (A), duloxetine (B) and mexiletine (C) on paclitaxel-induced mechanical allodynia in mice. Each symbol represents the mean PWT for 10 mice. Drugs were administered orally once a day for two weeks from day 8. ## $P < 0.01$ (vs. Normal, Wilcoxon test), * $p < 0.05$, ** $p < 0.01$ (vs. Control, Wilcoxon test [A], Steel test [B, C])

Fig. 3. Effect of etodolac, pregabalin, duloxetine and mexiletine on PWT of normal mice. Each symbol represents the mean PWT for 10 mice. Drugs were administered orally once a day for a week from day 0. * $p < 0.05$ (vs. Normal, Steel test).

Fig. 4. Effect of etodolac on antiproliferative activity of paclitaxel against A549 (A),
MDA-MB-468 (B) and OVCAR (C) cells. Each symbol represents the mean of triplicate values of the cell viability relative to untreated cells.

Fig. 5. Distribution of etodolac and diclofenac in central and peripheral nervous tissues of paclitaxel-treated mice. $^{14}$C-Etodolac (10 mg/kg) or $^{14}$C-diclofenac (3 mg/kg) was orally administered. Bars represent the mean tissue-to-plasma radioactivity ratio for three mice.

Fig. 6. Histopathology of peripheral nervous tissue in normal and paclitaxel-treated mice. Photomicrographs of nerve-tissue sections from an untreated mouse (A, D, G), a paclitaxel-treated mouse (B, E, H), and a mouse treated with both etodolac and paclitaxel (C, F, I). The green arrows show vacuoles and the red arrowheads show degenerated axons. A–C, dorsal root; D–I, sciatic nerve; A–F, hematoxylin-eosin stain; G–I, semi-thin specimens stained with toluidine blue. Bars = 50 μm
Table 1

Radioactivity in tissues after oral administration of $^{14}$C-etodolac (10 mg/kg) or $^{14}$C-diclofenac (3 mg/kg) to normal and paclitaxel-treated mice.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Normal mice</th>
<th>Paclitaxel-treated mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Radioactivity level (ng equivalent /g or mL)</td>
<td>Radioactivity level (ng equivalent /g or mL)</td>
</tr>
<tr>
<td></td>
<td>Tissue/plasma (%)</td>
<td>Tissue/plasma (%)</td>
</tr>
<tr>
<td>Plasma</td>
<td>13400</td>
<td>22800</td>
</tr>
<tr>
<td></td>
<td>(100.0)</td>
<td>(100.0)</td>
</tr>
<tr>
<td>Cerebrum</td>
<td>246</td>
<td>304</td>
</tr>
<tr>
<td></td>
<td>(1.8)</td>
<td>(1.3)</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>400</td>
<td>480</td>
</tr>
<tr>
<td></td>
<td>(3.0)</td>
<td>(2.1)</td>
</tr>
<tr>
<td>Sciatic nerve</td>
<td>2620</td>
<td>5020</td>
</tr>
<tr>
<td></td>
<td>(19.6)</td>
<td>(22.0)</td>
</tr>
<tr>
<td>DRG</td>
<td>3090</td>
<td>5590</td>
</tr>
<tr>
<td></td>
<td>(23.1)</td>
<td>(24.5)</td>
</tr>
</tbody>
</table>

Each value represents the mean radioactivity level for three mice. The figures in parentheses represent the tissue concentration as a percentage of the plasma concentration.
Table 2

Effect of etodolac on paclitaxel-induced pathological changes in dorsal root, dorsal cord and sciatic nerve.

<table>
<thead>
<tr>
<th>Group</th>
<th>Normal</th>
<th>Control</th>
<th>Etodolac</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of animals</td>
<td>4</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Vacuolization, axonal degeneration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dorsal root</td>
<td>4/0/0/0/0</td>
<td>0/2/3/0/0*</td>
<td>0/2/3/0/0</td>
</tr>
<tr>
<td>dorsal cord</td>
<td>4/0/0/0/0</td>
<td>3/2/0/0/0</td>
<td>4/1/0/0/0</td>
</tr>
<tr>
<td>sciatic nerve</td>
<td>4/0/0/0/0</td>
<td>1/4/0/0/0*</td>
<td>0/5/0/0/0</td>
</tr>
</tbody>
</table>

The severity of the histopathological changes was classified as −, normal; ±, minimal; +, mild; ++, moderate; or ++++, marked. The data show the numbers of mice with each grade as follows: −/±/+/++/++++. *p < 0.05 (vs. Normal, Wilcoxon test)
Table 3

Effect of etodolac on paclitaxel-induced pathological changes in sciatic nerves determined by examination of semi-thin specimens.

<table>
<thead>
<tr>
<th>Group</th>
<th>Normal</th>
<th>Control</th>
<th>Etodolac</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of animals</td>
<td>4</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Number of degenerating nerves/mm²</td>
<td>39.7±26.6</td>
<td>191.8±169.8*</td>
<td>135.1±100.8</td>
</tr>
</tbody>
</table>

The values show the mean±SD of the number of degenerating nerves per unit area of sciatic nervous tissue. * p < 0.05 (vs. Normal, Wilcoxon test)
Figure 1B

- Normal
- Control
- Diclofenac 3 mg/kg

PWT (g)

Day 0  
Day 8  
Day 15  
Day 22

Time after paclitaxel administration

Pre 0.5 h 4 h  
Pre 0.5 h 4 h  
Pre 0.5 h

# # # #

# # # #

# #

# #
Figure 2A

Graph showing the effect of paclitaxel administration on PWT (g) over different time points and conditions.

- **Normal**
- **Control**
- **Pregabalin 30 mg/kg**

Time points:
- **Day 0**
- **Pre 0.5 h**
- **4 h**

Day 8:
- Pre 0.5 h
- Pre 4 h

Day 15:
- Pre 0.5 h
- Pre 4 h

Day 22:
- Pre 0.5 h
- Pre 4 h

Significance markers: ##, **
Figure 2B

Time after paclitaxel administration

- Normal
- Control
- Duloxetine 30 mg/kg
- Duloxetine 100 mg/kg
Figure 3
Figure 4A

Relative cell viability

Concentration of etodolac (μM)

- Untreated
- Paclitaxel 3 nM
- Paclitaxel 6 nM
Figure 4B

Graph showing the relative cell viability against concentration of etodolac (μM). The graph compares untreated cells and those treated with 2.5 nM and 5 nM paclitaxel. The y-axis represents relative cell viability, and the x-axis represents concentration of etodolac (μM).
Figure 5

- Single dose etodolac
- Single dose diclofenac
- Multiple doses etodolac
- Multiple doses diclofenac

Tissue/Plasma (%) vs. Tissue Types:

- Cerebrum
- Spinal cord
- Sciatic nerve
- DRG

Central nervous tissue vs. Peripheral nervous tissue.
Figure 6