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Original Article

Trametinib suppresses chemotherapy-induced cold and mechanical allodynia via inhibition of extracellular-regulated protein kinase 1/2 activation

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Abstract: Chemotherapy-induced neuropathy is a common, dose-dependent adverse effect of some anti-cancer drugs and leads to discontinuation of chemotherapy and detrimental dose reductions, thereby affecting the quality of life of cancer patients. Currently, no treatment can effectively prevent or treat chemotherapy-induced neuropathy. Therefore, understanding its underlying molecular mechanisms may help to identify novel therapies for treating it. Some disease-induced neuropathy involve the activation of mitogen-activated protein kinases (MAPKs), such as extracellular-regulated protein kinase 1/2 (ERK1/2). In the present study, we investigated whether ERK1/2 inhibition can prevent chemotherapy-induced neuropathy. We found that trametinib, an MEK inhibitor, suppressed oxaliplatin-, paclitaxel-, vincristine-, and bortezomib-induced cold and mechanical allodynia in mice. In addition, treatment with oxaliplatin, paclitaxel, vincristine, or bortezomib enhanced ERK1/2 and c-Jun N-terminal kinase (JNK) phosphorylation in the spinal cord lumbar segments 4-6, and when combined with trametinib, can prevent chemotherapy-induced neuropathy via the suppression of ERK1/2 activation, but does not affect JNK activation. In conclusion, we demonstrated that the disruption of this pathway by MEK inhibitors suppresses oxaliplatin-, paclitaxel-, vincristine-, and bortezomib-induced neuropathy. This suggests that inhibition of the MEK/ERK pathway could prevent chemotherapy-induced neuropathy and MEK inhibitors could be used in combination with anti-tumor drugs during pharmacotherapy.

Keywords: ERK1/2, trametinib, chemotherapy-induced neuropathy

Introduction

Chemotherapy-induced neuropathy is a common and potentially dose-limiting side effect of many chemotherapy treatment regimens [1]. The prevalence rate of chemotherapy-induced neuropathy was 68.1% within the first month of treatment [2] and varies from 10% to 100% depending on the chemotherapy, regimen dose, and patient situation [3]. A high prevalence of chemotherapy-induced neuropathy has significantly decreased the quality of life and has often resulted in the discontinuation of chemotherapy, which may ultimately affect overall survival. However, to date, no studies have reported method for preventing chemotherapy-induced neuropathy [3, 4]. This has generated a large unmet medical need for novel agents to improve relief of chemotherapy-induced neuropathy.

The activation of mitogen-activated protein kinases (MAPKs), including extracellular-regulated protein kinase 1/2 (ERK1/2), p38MAPK, and c-Jun N-terminal kinase (JNK), can contribute to chemotherapy-induced neuropathy. Oxaliplatin induces apoptosis through the activation of ERK1/2 in rat dorsal root ganglion (DRG) cells, and the protein kinase C (PKC)/ERK pathway in the spinal cord and brain is activated during cold and mechanical allodynia [5-7]. Phosphorylated ERK1/2 and p38MAPK levels in spinal cord and spinal microglia are correlated with paclitaxel-induced neuropathy [8], and in rats, treatment with paclitaxel evoked mechanical hypersensitivity via increased

ERK1/2 and JNK activation in the spinal cord [9]. In glia-mediated neuroinflammation, vincristine induced the activation of glial cells; phosphorylation of ERK1/2, JNK, and p38-MAPK; and production of inflammatory cytokine in the spinal cord. This suggests that vincristine produces mechanical hypersensitivity [10]. It was also demonstrated that the administration of bortezomib induced mechanical hypersensitivity through upregulation of the expression of tumor necrosis factor α and phosphorylated JNK in the DRG of rats [11]. Furthermore, activation of MAPKs modulated activities of ion channels, such as sodium channel Nav1.7, Nav1.8, and transient receptor potential (TRP) vanilloid 1 (TRPV1), which have also been reported to contribute to chemotherapy-induced neuropathy [12-16]. Moreover, activation of ERK1/2 in spinal cord was observed in allodynia and hyperalgesia [17], and noxious stimuli-induced ERK1/2 phosphorylation has been studied in numerous animal pain models [18]. Additionally, ERK1/2 is thought of as involved with the mechanisms of neuropathic pain and may be targeted for therapy

Trametinib is a highly selective allosteric inhibitor of MAPK kinase (MEK) 1/2. It inhibits ERK1/2 phosphorylation [19]. In clinical settings, a BRAF inhibitor composed of trametinib and dabrafenib is widely used for treating and preventing metastatic melanoma [20]. Cetuximab, an anti-epidermal growth factor receptor (EGFR) antibody, in combination with trametinib, an MEK1/2 inhibitor that is used for treating colorectal cancer by targeting the NRAS mutant gene, underscores the importance of therapeutic intervention against the MEK/ERK and EGFR pathways to achieve maximal therapeutic efficacy in colorectal cancer harboring NRAS mutations [21]. Trametinib enhances the sensitivity to phosphoinositide 3-kinase inhibitors in triple negative breast cancer [22]. Because it is already clinically used, trametinib could also be used for suppressing chemotherapy-induced neuropathy. Therefore, we investigated whether the MEK inhibitor trametinib suppresses chemotherapy-induced neuropathy in a mouse model.

Materials and methods

Mice

Male Balb/c mice (age, 5 weeks) were purchased from Shimizu Laboratory Animals (Kyoto, Japan). The mice were maintained in an environment of 25°C under controlled lighting (12-h light/12-h dark cycle) and allowed free access to water and food pellets. All animal studies and protocols were approved by Kindai University Animal Care and Use Committee.

Drugs

Trametinib, oxaliplatin, and Bortezomib were purchased from LC Laboratories (Woburn, MA, USA). Paclitaxel and vincristine were purchased from Wako (Osaka, Japan). Trametinib, paclitaxel, vincristine, and bortezomib were dissolved in saline containing 0.5% dimethyl sulfoxide (DMSO). Oxaliplatin was dissolved in 5% glucose solution.

Oxaliplatin, paclitaxel, vincristine, and bortezomib-induced allodynia models

To measure the cold and mechanical sensitivity, mice were treated with (Day 0 and 7), oxaliplatin (6 mg/kg), paclitaxel (6 mg/kg), vincristine (0.2 mg/kg), bortezomib (1 mg/kg), or vehicle (saline) on Day 0 and 7 (n = 10 for each group). On Day 0, mice were treated with trametinib, 12 h after the administration of oxaliplatin, paclitaxel, vincristine, or bortezomib. Trametinib was administered orally (p.o.) at 0.5 mg/kg daily from Day 0 to 14 (n = 10 for each group). Behavioral tests were performed from Day 0 to 14.

Behavioral assays

Behavioral assays were performed as described in a previous study [6]. Cold sensitivity was assessed with the hot/cold-plate analgesimeter (Ugo Basile, Milan, Italy). Each mouse was placed on the center of a plate maintained at 10°C (cold allodynia); chemotherapy-induced pain-related behaviors, such as lifting and licking of the hind paw, were observed and the time was recorded (cut-off time at 30 s).

Mechanical allodynia and hyperalgesia were investigated using 0.16, 0.4, and 1.4 g of von Frey filaments (Ugo Basile). For each filament, five stimuli were applied at an interval of 3-5 s, and mechanical sensitivity was scored as follows: 0, no response; 1, paw withdrawal; or 2, immediate flinching of the stimulated paw. Paw withdrawal threshold of five trials from both hind paws of each mice were averaged and recorded as mean ± S.E.M.

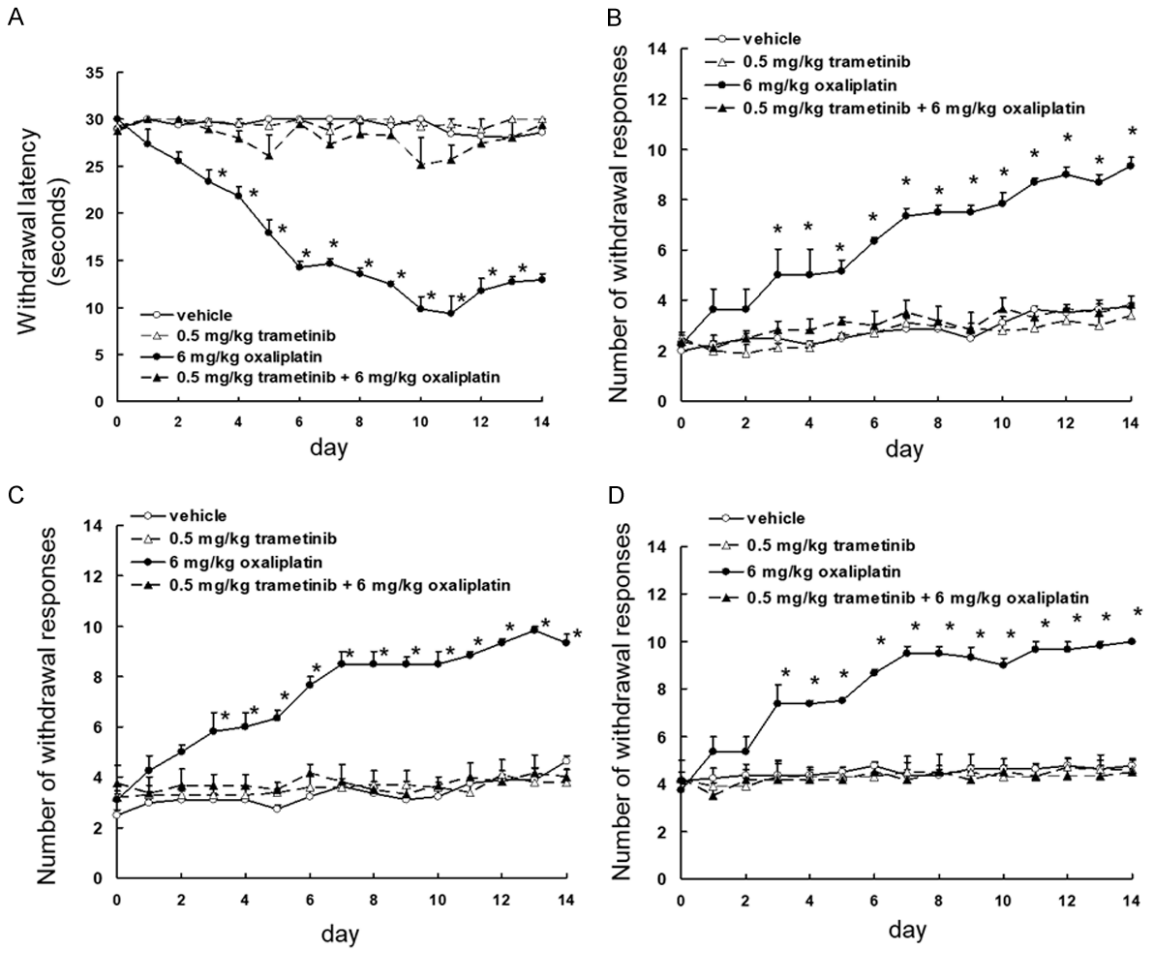

Figure 1. Trametinib inhibited oxaliplatin-induced cold and mechanical allodynia. Oxaliplatin (6 mg/kg, n = 10) was administered i.p. weekly for 2 weeks (days 0 and 7). Trametinib (0.5 mg/kg, n = 10) was administered p.o. daily for 14 days. (A) Withdrawal latencies, presented as means ± S.E.M., represent the time it took the mice to withdraw their hind paws following cold stimulation (10°C). *P < 0.01 vs. vehicles (ANOVA with Dunnet's test). (B-D) Number of paw lifts elicited by five mechanical stimulations using von Frey filaments corresponding to (B) innocuous (0.16 g), (C) intermediate (0.4 g), and (D) noxious (1.4 g) bending forces. The pain threshold is obtained for two paw lifts. *P < 0.01 vs. vehicles (ANOVA with Dunnet's test).

Luminex assay

The lumber spinal cords were homogenized in ice-cold buffer and proteins were extracted. The supernatants were examined using a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Protein phosphorylation of ERK1/2 (Thr185/Tyr187), JNK(Thr183/Tyr185), nuclear factor κB (NF-κB) (Ser536), CREB (Ser133), and p38MAPK (Thr180/Tyr182) was determined with the 9-plex Multi-Pathway Magnetic Bead Panel (#46-680MAG, Merck Millipore, Nottingham, UK) following the manufacturer's protocol. β-Tubulin beads (#64-713MAG, Merck Millipore) was added to correct for protein load.

Western blotting

The protein extract of the lumber spinal cords in mice was obtained and western blotting assay was performed as previously described [6]. The supernatants of protein extract were examined using a BCA protein assay kit (Thermo Scientific). The protein extracts (20 μg) were fractionated uisng SDS-PAGE and transferred to PVDF membranes (GE Healthcare, Buckinghamshire, UK). The membranes were blocked with a solution containing 3% skim milk and incubated overnight at 4°C with each of the following antibodies: anti-phospho-ERK1/2 (Thr202/Tyr204), anti-ERK1/2 (Cell Signaling Technology, Beverly, MA, USA), and anti-β-actin

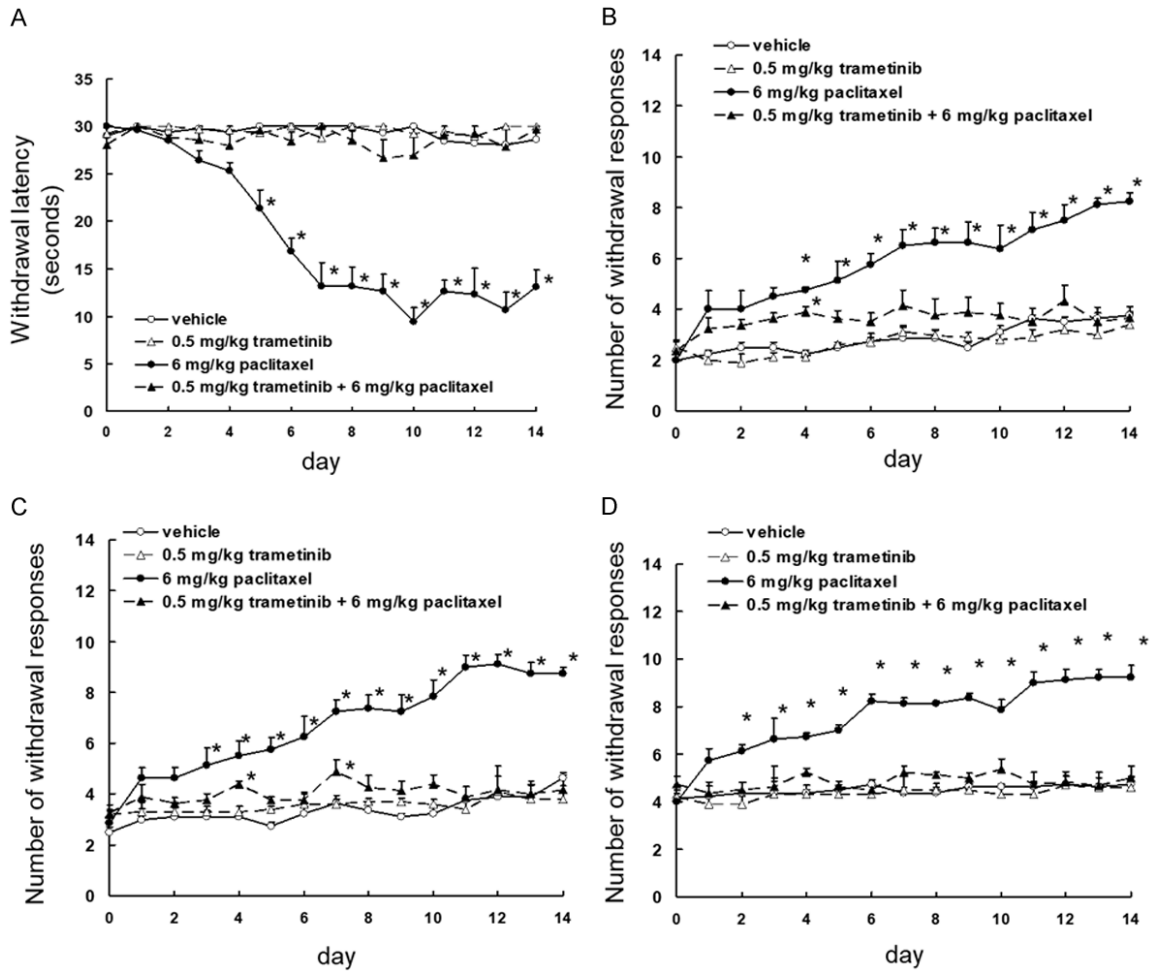

Figure 2. Trametinib inhibited paclitaxel-induced cold and mechanical allodynia. Paclitaxel (6 mg/kg, n = 10) was administered i.p. weekly for 2 weeks (days 0 and 7). Trametinib (0.5 mg/kg, n = 10) was administered p.o. daily for 14 days. (A) Withdrawal latencies, presented as means ± S.E.M., represent the time it took the mice to withdraw their hind paws following cold stimulation (10°C). *P < 0.01 vs. vehicles (ANOVA with Dunnet's test). (B-D) Number of paw lifts elicited by five mechanical stimulations using von Frey filaments corresponding to (B) innocuous (0.16 g), (C) intermediate (0.4 g), and (D) noxious (1.4 g) bending forces. The pain threshold is obtained for two paw lifts. *P < 0.01 vs. vehicles (ANOVA with Dunnet's test).

antibody (Sigma, St. Louis, MO, USA). Then, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG sheep antibodies (GE Healthcare) for 1 h at room temperature. The reactive proteins were visualized using Luminata Forte (Merck Millipore) according to the manufacturer's instructions.

Statistics

All results are expressed as means and S.E.M. of several independent experiments. Statistical comparisons were performed by analysis of variance (ANOVA) with Dunnett's test for multiple comparisons. *P* values less than 5% were regarded as significant.

Results

Trametinib suppresses chemotherapy-induced neuropathy

To evaluate the protective effect of trametinib against oxaliplatin-, paclitaxel-, vincristine-, or bortezomib-induced neuropathy, we administrated 0.5 mg/kg of trametinib daily to mice that received oxaliplatin, paclitaxel, vincristine, or bortezomib, 1 week apart (days 0 and 7). Oxaliplatin, paclitaxel, vincristine, and bortezomib induced a significant progressive reduction in withdrawal thresholds at 10°C (**Figures 1A**, **2A**, **3A**, and **4A**, respectively). Oral administration of trametinib significantly suppressed

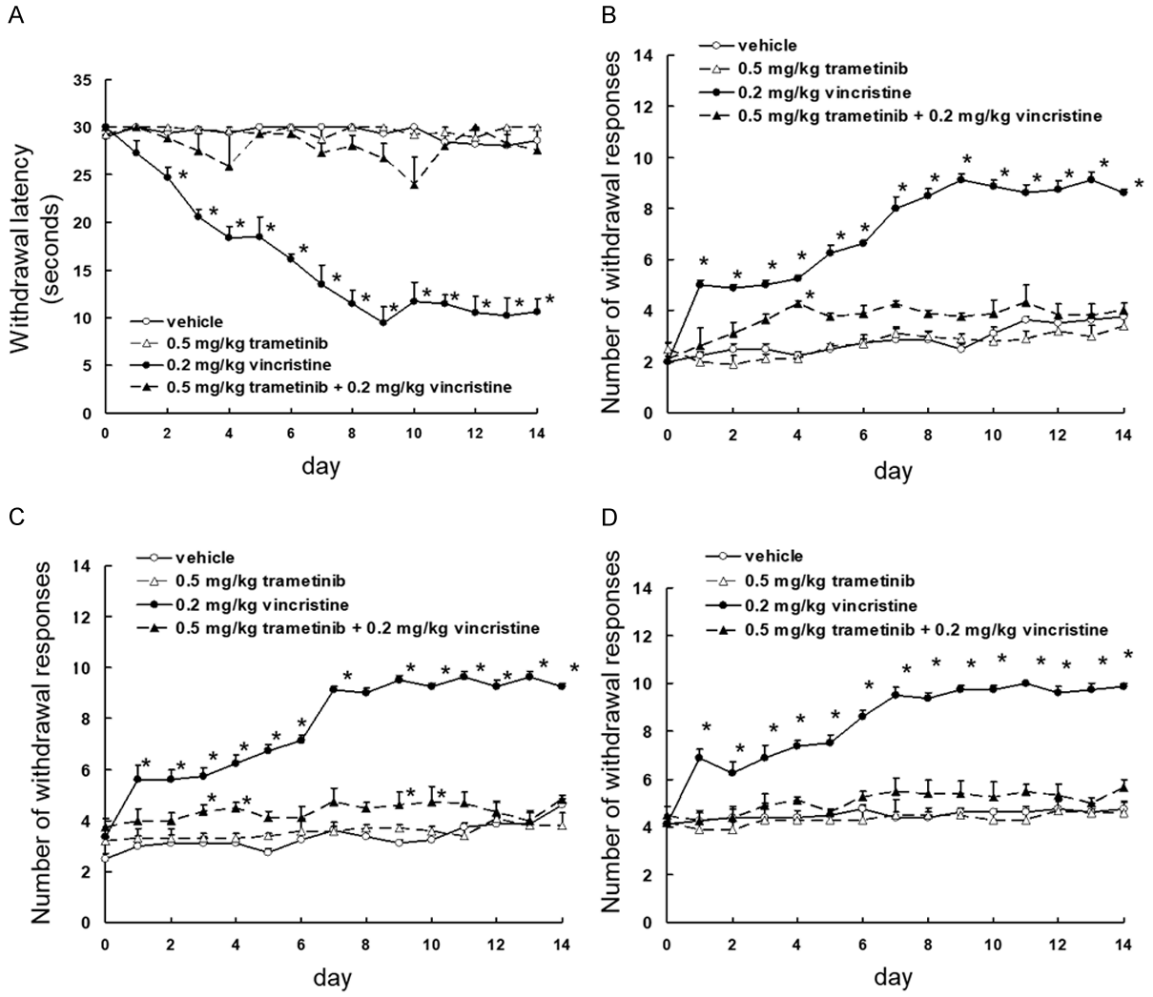

Figure 3. Trametinib inhibited vincristine-induced cold and mechanical allodynia. Vincristine (0.2 mg/kg, n = 10) was administered i.p. weekly for 2 weeks (days 0 and 7). Trametinib (0.5 mg/kg, n = 10) was administered p.o. daily for 14 days. (A) Withdrawal latencies, presented as means ± S.E.M., represent the time it took the mice to withdraw their hind paws following cold stimulation (10°C). *P < 0.01 vs. vehicles (ANOVA with Dunnet's test). (B-D) Number of paw lifts elicited by five mechanical stimulations using von Frey filaments corresponding to (B) innocuous (0.16 g), (C) intermediate (0.4 g), and (D) noxious (1.4 g) bending forces. The pain threshold is obtained for two paw lifts. *P < 0.01 vs. vehicles (ANOVA with Dunnet's test).

oxaliplatin-, paclitaxel-, vincristine-, and bortezomib-induced cold allodynia (**Figures 1A**, **2A**, **3A**, and **4A**, respectively). In addition, trametinib inhibited oxaliplatin-, paclitaxel-, vincristine-, and bortezomib-induced withdrawal response in the von Frey test (0.14, 0.4, and 1.4 g respectively) (**Figures 1B-D**, **2B-D**, **3B-D**, and **4B-D**). Mice, which received oxaliplatin, paclitaxel, vincristine, bortezomib, and trametinib, did not exhibit any weight loss (Supplementary Figure 1). These observations suggest that trametinib suppressed oxaliplatin-, paclitaxel-, vincristine-, and bortezomib-induced neuropathy.

Trametinib inhibited chemotherapy-induced phosphorylation of ERK1/2 expression

The initial screening of signal transduction molecules involved in oxaliplatin-, paclitaxel-, vincristine-, and bortezomib-induced neuropathy was performed using the Luminex assay in the spinal cord (lumber segments 4-6). Oxaliplatin, paclitaxel, vincristine, and bortezomib induced the activation of ERK1/2 and JNK, but not p38MAPK, NF-κB, and CREB (**Figure 5A**). Trametinib inhibited the expression of phosphorylated ERK1/2 (phospho-ERK1/2), but not JNK (**Figure 5A**).

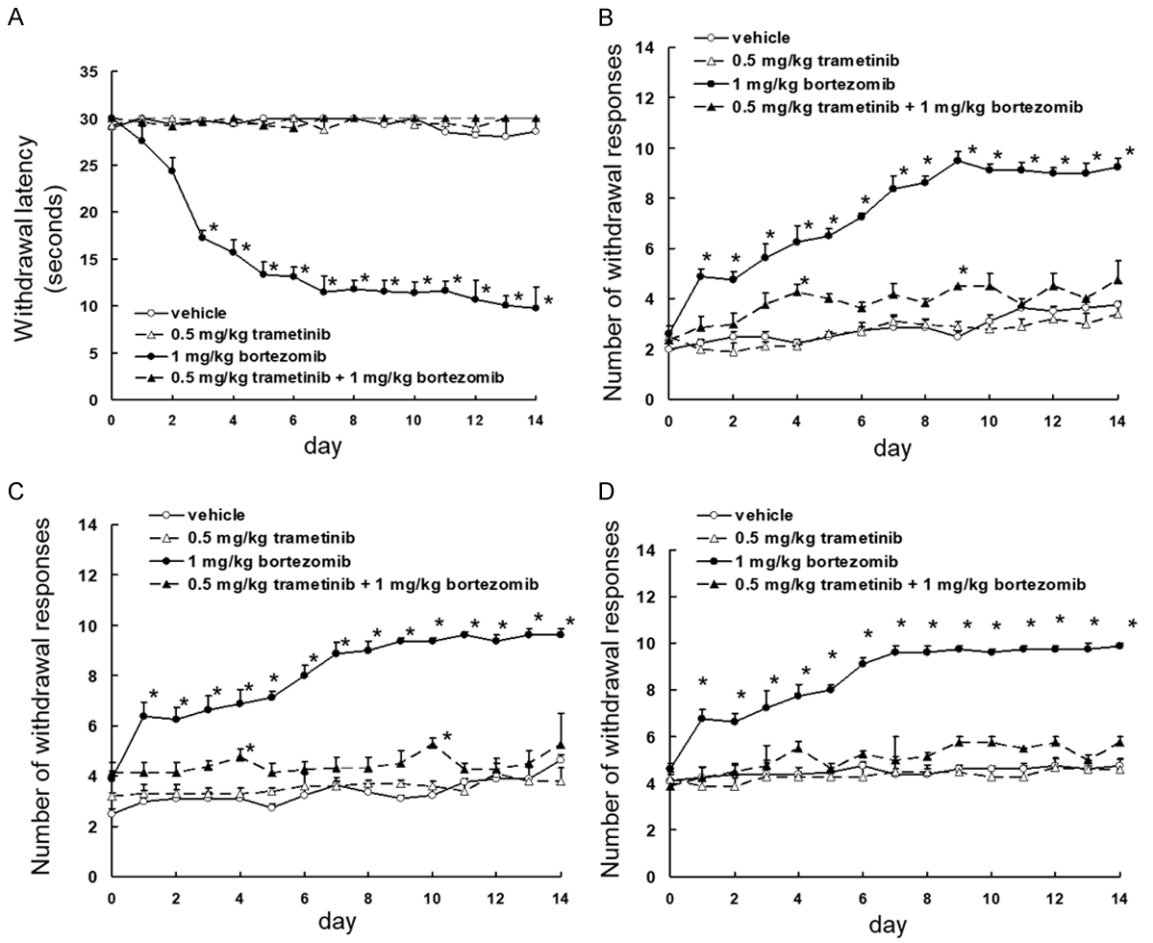

Figure 4. Trametinib inhibited bortezomib-induced cold and mechanical allodynia. Bortezomib (1 mg/kg, n = 10) was administered i.p. weekly for 2 weeks (days 0 and 7). Trametinib (0.5 mg/kg, n = 10) was administered p.o. daily for 14 days. (A) Withdrawal latencies, presented as means ± S.E.M., represent the time it took the mice to withdraw their hind paws following cold stimulation (10°C). *P < 0.01 vs. vehicles (ANOVA with Dunnet's test). (B-D) Number of paw lifts elicited by five mechanical stimulations using von Frey filaments corresponding to (B) innocuous (0.16 g), (C) intermediate (0.4 g), and (D) noxious (1.4 g) bending forces. The pain threshold is obtained for two paw lifts. *P < 0.01 vs. vehicles (ANOVA with Dunnet's test).

Next, using western blotting, we confirmed the expression of phospho-ERK1/2 in the lumbar spinal cord. A marked increase in the expression of phospho-ERK1/2 was observed in mice treated with oxaliplatin, paclitaxel, vincristine, or bortezomib. Treatment with trametinib suppressed ERK1/2 activation because of oxaliplatin, paclitaxel, vincristine, or bortezomib (**Figure 5B**). These results indicate that the inhibitory effects of trametinib on oxaliplatin-, paclitaxel-, vincristine-, or bortezomib-induced neuropathy are expected through the suppression of the MEK/ERK pathway.

Discussion

In the present study, we demonstrated that trametinib suppresses oxaliplatin-, paclitaxel-, vincristine-, and bortezomib-induced neuropathy through the inhibition of the MEK/ERK pathway. Oxaliplatin-induced neuropathy involves the PKC activation within the spinal cord, thalamus, and periaqueductal area in the brain [6, 7]. In addition, paclitaxel-induced neuropathic pain involves the activation of PKCβII, PKCδ, and PKCε in the DRG of mice [23]. It was also reported that bortezomib-induced neuropathy correlates with the activation of glutamate *N*-methyl-D-aspartate receptor via PKC in the spinal cord of rats [24]. The activation of PKCα, PKCδ, and PKCε induced ERK1/2 phosphorylation in lumbar segments of mouse spinal cord [6]. Moreover, the present study is the first to present evidence of the phosphorylation of ERK1/2 in the spinal cord during bortezomib-induced neuropathy. These findings suggest

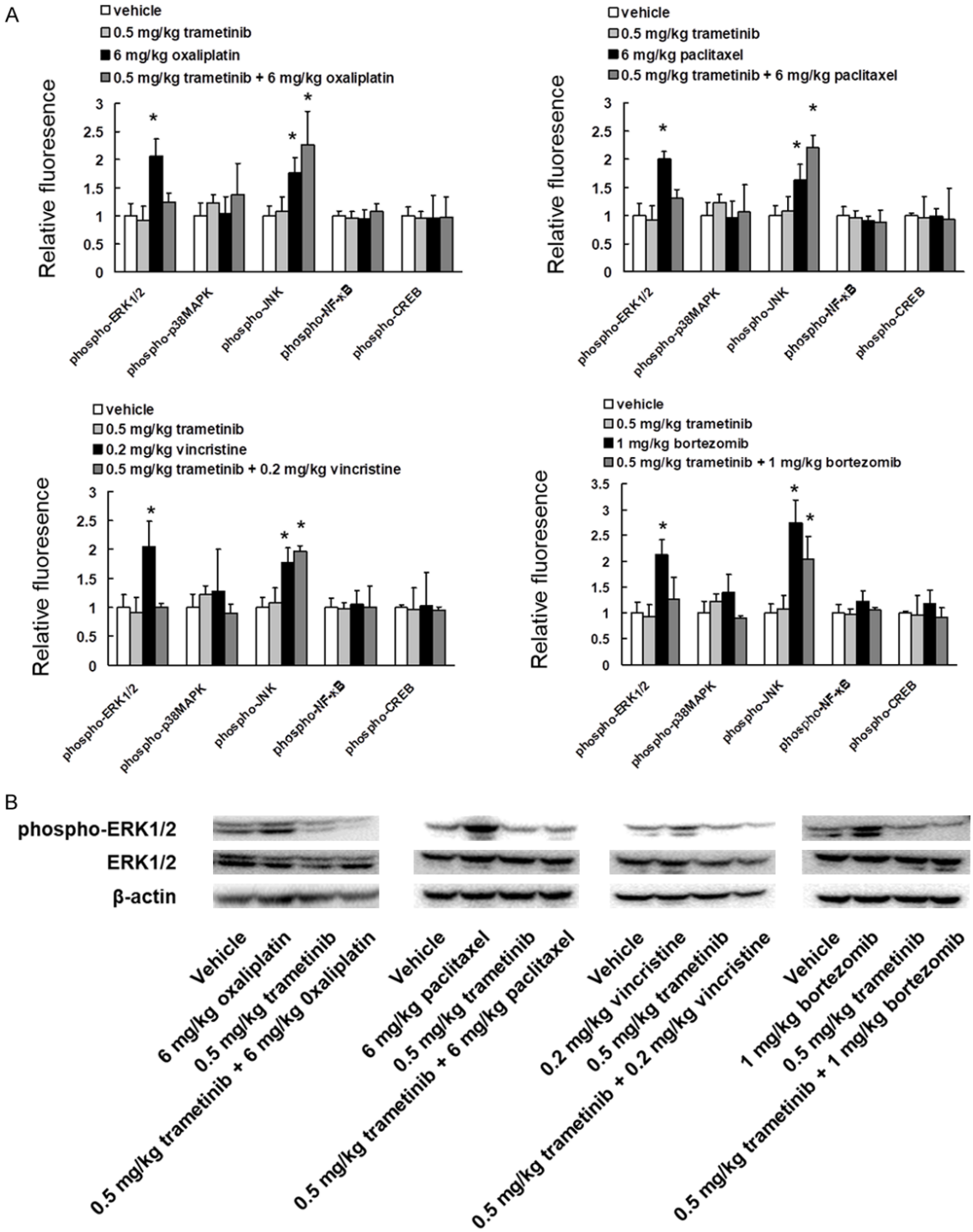

Figure 5. Trametinib inhibited the oxaliplatin-, paclitaxel-, vincristine-, and bortezomib-induced the activation of ERK1/2. (A) Spinal cords were lysed and phosphorylation of ERK1/2, p38MAPK, JNK, NF-κB, or CREB protein were measured by Luminex assay. (B) phosphorylated ERK1/2 (phospho-ERK1/2) protein content analyzed by western blotting in the spinal cord (L4-L6) obtained from mice on day 14 after treatment with oxaliplatin, paclitaxel, vincristine, bortezomib or trameinib. Loading of equivalent amounts of protein was verified by the relative expression of β-actin.

that chemotherapy-induced neuropathy involves the ERK1/2 activation and that its inhibition may be beneficial for preventable chemotherapy-induced neuropathy.

Several members of the TRP family of receptors are implicated in chemotherapy-induced neuropathy. It was reported that the treatment of cultured DRG neurons with oxaliplatin increases the expression of TRPV1, TRPA1, and TRPM8 mRNA, and oxaliplatin-induced cold allodynia correlates with the upregulation of TRPA1 and TRPM8 in mice and rats [25, 26]. In addition, paclitaxel-induced cold and mechanical allodynia is associated with increased TRPA1 and TRPV1 expression in the DRG neurons of mice, rats, and humans [27, 28]. It has also been indicated that the up-regulation of TRPV1 contributes to vincristine-induced mechanical allodynia and that TRPA1 antagonist HC-030031 inhibits bortezomib- and oxaliplatin-induced cold and mechanical allodynia [29, 30]. These findings suggest that the activation and/or upregulation of members of the TRP family is important in chemotherapy-induced neuropathy. It was reported that ERK1/2 activation increases TRPV1 expression in DRG neurons [31]. Furthermore, a study reported that interleukin-1α increased TRPA1 expression via ERK1/2 activation [32]. PKC/ERK pathway activation by nerve growth factors promotes the sensitization of TRPV1 in DRG neurons [33]. Moreover, TRPV1, TRPA1, and TRPM8 activation enhances the transient levels of intracellular Ca^{2+}, leading to ERK activation [34, 35]. Altogether, these findings suggest that ERK1/2 and members of the TRP family, such as TRPV1, TRPA1, and TRPM8, interact with each other, which may affect nerve sensitivity during chemotherapy.

In this study, we found that the orally administered trametinib (0.5 mg/kg) suppressed oxaliplatin-, paclitaxel-, vincristine-, and bortezomib-induced neuropathy. It has been previously reported that the C_{max} following 0.5 mg/kg trametinib administration, was approximately 70 ng/mL in mice [36]. However, the C_{max} of trametinib following a daily oral administration of 2.5 mg/day was 63.2 ng/mL in cancer patients [37]. A comparison of trametinib plasma concentrations in mice and humans showed that the C_{max} in humans was similar to that of mice. These findings suggest that treatment with trametinib in mice or humans may achieve commensurate plasma drug concentrations.

In conclusion, we provide the first evidence of effective inhibition of oxaliplatin-, paclitaxel-, vincristine-, and bortezomib-induced neuropathy by trametinib. Our findings indicate that oxaliplatin-, paclitaxel-, vincristine-, and bortezomib-induced neuropathy is involved in MEK/ERK pathway activation, which effectively suppresses chemotherapy-induced neuropathy. Therefore, MEK inhibitors, such as trametinib, may be therapeutically beneficial for preventing chemotherapy-induced neuropathy.

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Disclosure of conflict of interest

None.

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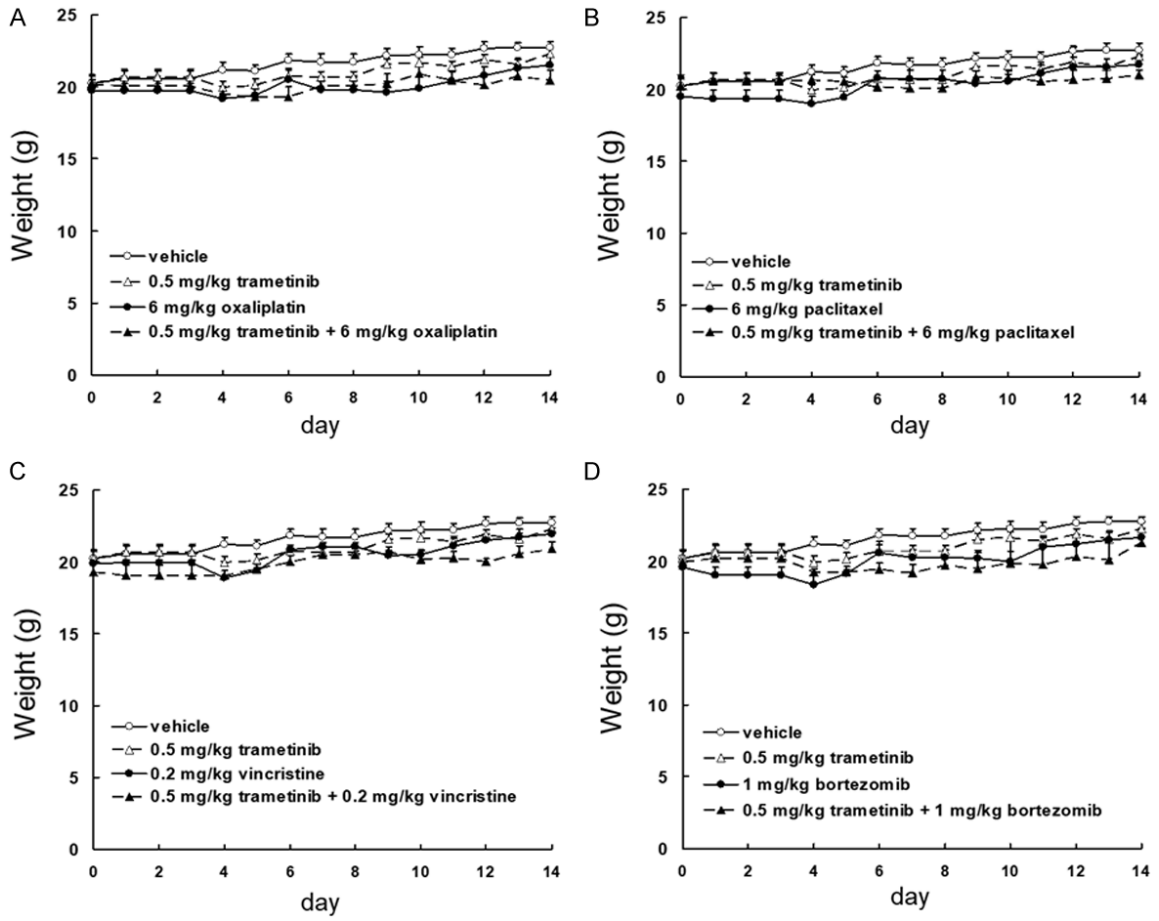

Supplementary Figure 1. Co-treatment with trametinib and oxaliplatin, paclitaxel, vincristine, or bortezomib did not affect body weight in mice. Safety of oxaliplatin, paclitaxel, vincristine, or bortezomib and trametinib administrated in vivo. (A) Oxaliplatin (6mg/kg, n = 10), (B) Paclitaxel (6 mg/kg, n = 10), (C) vincristine (0.2 mg/kg, n = 10), or (D) bortezomib (1 mg/kg, n = 10) were administrated i.p. weekly for 2 weeks (days 0 and 7). Trametinib (0.5 mg/kg, n = 10) was administrated p.o. daily for 14 days. Mice were weighed before the first treatment and daily for the duration of treatment. Means and S.E.M. are shown.