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TRPM3 as a novel target to alleviate acute oxaliplatin-induced peripheral neuropathic pain

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Abstract

Chemotherapy-induced peripheral neuropathic pain (CIPNP) is an adverse effect observed in up to 80% of patients of cancer on treatment with cytostatic drugs including paclitaxel and oxaliplatin. Chemotherapy-induced peripheral neuropathic pain can be so severe that it limits dose and choice of chemotherapy and has significant negative consequences on the quality of life of survivors. Current treatment options for CIPNP are limited and unsatisfactory. TRPM3 is a calcium-permeable ion channel functionally expressed in peripheral sensory neurons involved in the detection of thermal stimuli. Here, we focus on the possible involvement of TRPM3 in acute oxaliplatin-induced mechanical allodynia and cold hypersensitivity. In vitro calcium microfluorimetry and whole-cell patch-clamp experiments showed that TRPM3 is functionally upregulated in both heterologous and homologous expression systems after acute (24 hours) oxaliplatin treatment, whereas the direct application of oxaliplatin was without effect. In vivo behavioral studies using an acute oxaliplatin model for CIPNP showed the development of cold and mechano hypersensitivity in control mice, which was lacking in *TRPM3* deficient mice. In addition, the levels of protein ERK, a marker for neuronal activity, were significantly reduced in dorsal root ganglion neurons derived from *TRPM3* deficient mice compared with control after oxaliplatin administration. Moreover, intraperitoneal injection of a TRPM3 antagonist, isosakuranetin, effectively reduced the oxaliplatin-induced pain behavior in response to cold and mechanical stimulation in mice with an acute form of oxaliplatin-induced peripheral neuropathy. In summary, TRPM3 represents a potential new target for the treatment of neuropathic pain in patients undergoing chemotherapy.

Keywords: TRP channel, TRPM3, CIPNP, Neuropathic pain

1. Introduction

Antineoplastic chemotherapeutic regimes have proven tremendously efficient in the regression of previously untreatable cancers. Chemotherapy, however, is highly notorious for its toxic effects on the central nervous system (CNS) including the progressive deterioration of learning, memory, and other cognitive skills.[25,38] In addition, peripheral neuropathy is a potentially chronic and, in specific cases, also acute adverse event that occurs secondary to the treatment with various cytostatic drugs such as platinum-based compounds, taxanes, and vinca alkaloids. Neuropathic pain is a characteristic feature of peripheral neuropathy secondary to chemotherapy, which may be experienced in the form of paresthesia, burning, or tingling sensations in the peripheral extremities.[2,5] Overall, chemotherapy-induced peripheral neuropathic pain (CIPNP) has a significant negative effect on the quality of life of cancer survivors. Platinum-based compounds such as oxaliplatin and cisplatin have the strongest correlation with peripheral neuropathy.[1] Although most antineoplastic drugs lead to a delayed development of neuropathy, oxaliplatin and paclitaxel can induce an acute pain syndrome, which can become chronic after long-term treatment.

Several members of the transient receptor potential (TRP) superfamily of cation channels are functionally expressed at the sensory endings of dorsal root ganglion (DRG) neurons, where they have been implicated in the transmission of a diverse range of mechanical, chemical, and thermal sensory stimuli. With the presence of a leaky barrier between the blood and DRG neurons, it has been speculated that chronic exposure to antineoplastic drugs, in particular platinum-based compounds, could lead to a modified activity of TRP channels in sensory DRG neurons.[28] Especially, treatment with oxaliplatin triggers the upregulation of the nociceptors TRPV1, TRPA1, and TRPM8 in DRG neurons, which may contribute to oxaliplatin-induced thermal hypersensitivity.[3] Paclitaxel has been found to induce an increased release of substance P and other neuropeptides responsible for inducing hyperalgesia in the peripheral extremities.[29] The current options to prevent or treat CIPNP are narrow and rather limited to duloxetine[24] and tramadol.[18] Based on the relative abundance of TRP channels identified in DRG neurons, and their potential role in mediating

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CIPNP, TRP channels represent promising novel targets for treating chemotherapy-induced peripheral pain.[13,19] Recently, Transient Receptor Potential cation Channel subfamily M member 3 (TRPM3) was identified as a novel sensory TRP channel expressed at the molecular and functional level in a large subset of C and Aδ sensory neurons of mouse[35] and human.[33] TRPM3 is a polymodally gated calcium permeable ion channel which can be activated by different chemical substances such us the endogenous neurosteroid pregnenolone sulphate (PS) and by heat.[35] Furthermore, TRPM3 activation is well described to induce pain and stimulates the release of neuropeptides such as calcitonin gene-related peptide (CGRP).[8] In addition, TRPM3 is already well described to be involved in different types of pain-like inflammatory[35] neuropathic pain and spontaneous pain after nerve injury.[27] Hence, given the functional expression of TRPM3 in somatosensory neurons and its role as nociceptor channel, this study aims to clarify the potential role of TRPM3 in CIPNP and to investigate its potential as a target to alleviate chemotherapy-induced peripheral neuropathic pain.

2. Materials and Methods

2.1. Cell culture and transfection

HEK293T cells stably expressing murine *Trpm3* (HEK-mTRPM3) and nontransfected HEK293 (NT) were designed and cultured as described previously.[35] HEK293T cells stably transfected with human TRPM3 were developed and validated by SB Drug Discovery (Glasgow, United Kingdom). The human TRPM3 was stably expressed in HEK293T cells (HEK-hTRPM3) after induction with tetracycline (3 μg/mL). For transient transfection, cells were transfected with 2 μg of DNA 24 to 48 hours before measurement using TransIT transfection reagents (Mirus, Madison, WI). Dorsal root ganglion neurons from adult (postnatal weeks 8-12) mice were isolated and cultured as described elsewhere.[8]

2.2. Calcium imaging and electrophysiology

HEK293T cells stably expressing murineTRPM3 cells or DRG neurons were loaded with Fura-2-acetoxymethyl for 30 minutes at 37°C. Fluorescence was measured during alternating illumination at 340 and 380 nm using Eclipse Ti (Nikon, Europe BV) fluorescence microscopy system, and absolute calcium concentration was calculated from the ratio of the fluorescence signals at these 2 wavelengths ($R = F340/F380$) as $[Ca^{2+}] = Km \times (R-R_{min})/(R_{max}-R)$, where Km, R_{min}, and R_{max} were estimated from in vitro calibration experiments with known calcium concentrations.[31] The standard external solution contained (in mM) 150 NaCl, 10 HEPES, 2 $CaCl_2$, and 1 $MgCl_2$ (pH 7.4 with NaOH). Whole-cell patch-clamp experiments were performed with an EPC-10 amplifier and PatchMasterPro Software (HEKA Elektronik, Lambrecht, Germany). The sampling rate for the current measurements was 20 kHz, and currents were digitally filtered at 2.9 kHz. The extracellular solution contained (in mM) 150 NaCl, 1 $MgCl_2$, and 10 HEPES (pH 7.4 with NaOH), and the standard internal solution contained (in mM) 100 CsAsp, 45 CsCl, 10 EGTA, 10 HEPES, and 1 $MgCl_2$ (pH 7.2 with CsOH).

2.3. Sensory nerve conduction

Sensory nerve conduction studies were performed as described previously.[30] Along the dorsal caudal nerve, sensory nerve action potentials (SNAPs) were measured by using platinum-coated intramuscular wire electrodes (Technomed Europe, Maastricht, The Netherlands) and a Natus UltraPro S100 (Natus Medical Incorporated, Pleasanton, CA).

2.4. RT–qPCR

Methods were based on established protocols from our research group.[34] RNA extraction of murine DRGs neurons was performed using RNeasy mini kit (QIAGEN). cDNA was generated from 1 μg of RNA using the First-strand cDNA Synthesis Kit (Thermofisher Scientific, Belgium). Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) was performed on triplicate cDNA samples using specific TaqMan gene expression assays in the StepOne PCR system. Data are represented as mean ± SEM of 2 $(-\Delta Ct)$ for which $\Delta Ct = Ct_{\text{gene of interest}} - Ct_{\text{geometric mean of endogenous controls}}$.

2.5. Animals

For all experiments, 8 to 12 week old male C57BL/6J (Janvier Labs, Le Genest-Saint-Isle, France) mice were used. To investigate the effects of TRPM3 deficiency, homozygous (*Trpm3*$^{-/-}$) mice were generated as described previously.[35] Mice of all genotypes were housed under identical conditions, with a maximum of 5 animals per cage, on a 12-hour light–dark cycle. Food and water were provided ad libitum.

2.6. Drugs

Oxaliplatin was purchased from Tocris Bioscience (United Kingdom) and freshly dissolved in 5% glucose solution. For the acute oxaliplatin model, mice received a single intraperitoneal administration of oxaliplatin (6 mg/kg) or vehicle.[4] Tramadol hydrochloride (5 mg/kg, Sigma, Belgium) was prepared in a mixture of 1% hydroxypropylmethylcellulose HPMC/0.5% Tween 80 and administered through oral gavage. Isosakuranetin (2 mg/kg) was dissolved in Miglyol 812 (Caesar & Loretz GmbH, Hilden, Germany) containing 0.1% dimethyl sulfoxide (DMSO), and compounds or the vehicle alone were injected intraperitoneally. Primidone and PS were purchased from Sigma-Aldrich (Belgium) and dissolved in DMSO. Stock solutions were 20 mM and 100 mM, respectively.

2.7. Ethical approval

All animal experiments were performed in accordance with the European Union Community Council guidelines and approved by the Local Ethics Committee of the KU Leuven (P108/2016).

2.8. Behavioral tests

2.8.1. Chemogenic pain model

PS (250 μM) was dissolved in PBS + 0.1% DMSO. The mice were allowed to acclimate in a clear plastic box for at least 40 min after which 20 μL of PS solution (5 nmol) was injected intraplantarly using a 30 G needle coupled to a Hamilton syringe, and behavior was recorded. Experiments were performed during the light cycle. The duration of the PS-evoked nocifensive behavior such as licking and flicking was quantified for 10 minutes.[35] Chemogenic pain model was actually performed on animals treated to vehicle and oxaliplatin at day −1 (baseline) and at day 6 after chemotherapy treatment.

2.8.2. Acetone spray test

Cold sensitivity was tested by the acetone spray test, as previously described.[11] In brief, 30 minutes before the start of the experiments, all the animals were placed in a testing apparatus consisting of 8 chambers with a mesh floor to acclimatize the mice with the environment. After the acclimation, a volume of 50 μL of acetone was applied to one of the hind paw and the responses were monitored for 60 seconds after application. Responses to a cold stimulation were graded according to the following 5-point scale: 0–no response; 1–brief lift, sniff, flick, or startle; 2–jumping and paw shaking; 3–multiple lifts and paw lick; 4–prolonged paw lifting, licking, shaking, or jumping; and 5–paw guarding.[11] Pain scores were determined by 2 independent researchers based on the post hoc analysis of video-recorded behavioral experiments, without knowledge of genotype or treatment. Scores from both researchers were equivalent and averaged to obtain the final pain score for statistical analysis.

2.8.3. Electronic Von Frey Assay

Mechanical allodynia was assessed by the von Frey test using an electronic device (Bioseb, France) as previously described.[15,21] Mice were placed individually in an 8-place chamber with a mesh floor and acclimatized for at least 30 minutes prior testing. Each paw was poked 4 times with a nonhygroscopic polypropylene von Frey tip of uniform diameter (0.8 mm), and the paw withdrawal threshold was calculated as the average of these 4 consecutive measurements. The numeric value of the force required to induce the paw withdrawal was recorded automatically by the apparatus.

2.9. Immunohistochemistry

Immunohistochemical staining of pERK on DRG neurons was performed based on a previously described protocol.[27] First, after euthanizing the animals by CO_2 inhalation, DRG neurons of each distinct group were dissociated, followed by their postfixation in 4% paraformaldehyde solution for 4 hours at 4°C and treated overnight at 4°C with the primary antibody (rabbit anti-pERK; 1:200, PhosphoSolutions Denver, CO). The pretreated sections were then incubated for 2 hours at room temperature with goat antirabbit conjugated to Cy3. Triple washing with PBS was performed between each step. Subsequently, the sections were fixed with 4′,6-diamidino-2-phenylindole (DAPI) mounting media (Sigma-Aldrich, Belgium). Immunofluorescence-labeled cells were imaged using a fluorescence microscope Eclipse Ci (Nikon). The NIH ImageJ software was used to quantify the labeled cells. Protein ERK (pERK) positive cells were defined as DAPI positive and a pERK immunofluorescence intensity that was above the threshold value of 2 times the mean value of the 5 lowest immunofluorescent pERK signals.

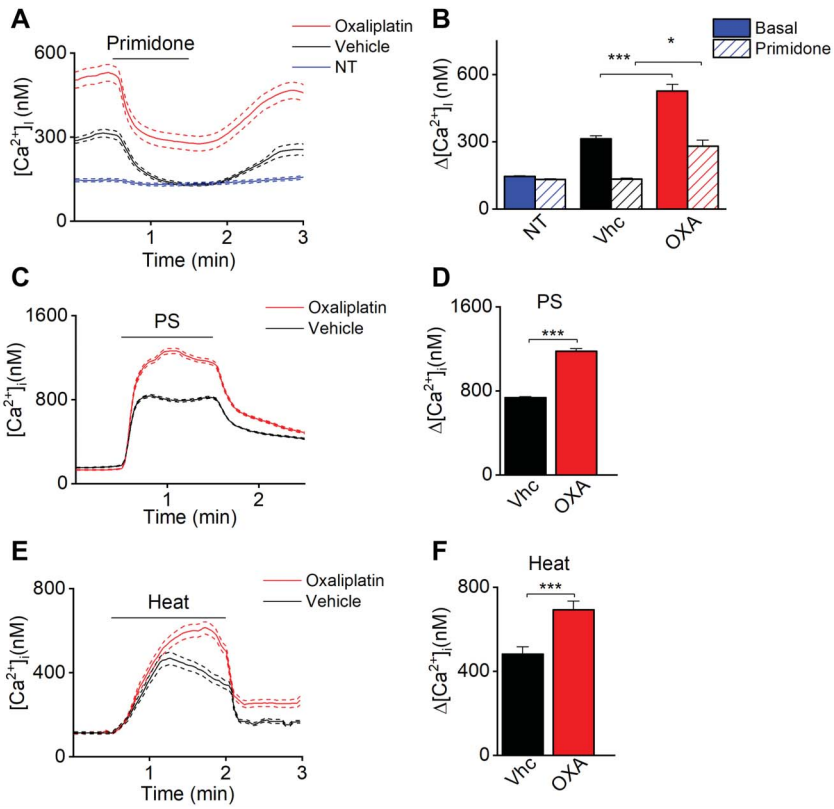

Figure 1. Modulation of TRPM3 function in HEK293T cells stably expressing murine TRPM3 after oxaliplatin pretreatment. (A) Time course of intracellular calcium concentrations ($[Ca^{2+}]_i$) (mean ± SEM) at 37°C on application of the TRPM3 inhibitors primidone (100 μM) in HEK-mTRPM3 cells after pretreatment with oxaliplatin (100 μM) (n = 299) and vehicle (n = 293) and nontransfected (NT) cells (n = 97) (N = 3 independent experiments). (B) Basal intracellular calcium concentrations before (full bars) and after the application of primidone (open bars). Data are represented as mean ± SEM. Statistically significant changes in the basal channel activity were assessed using a Kruskal–Wallis ANOVA with the Dunn post hoc test, where $^*P = <0.05$ and $^{***}P = <0.001$. (C and E) Time course of intracellular calcium concentrations ($[Ca^{2+}]_i$) (mean ± SEM) for HEK-mTRPM3 cells in response to PS (40 μM) and heat (37°C) after oxaliplatin (100 μM) and vehicle treatment. (D and F) Quantification of calcium responses for experiments as in panel (C and E), respectively. Where $^{***}P < 0.001$ (Mann–Whitney *U* test). ANOVA, analysis of variance; NT, nontransfected; PS, pregnenolone sulfate; Vhc, vehicle; OXA, oxaliplatin.

2.10. Experimental design and statistical analysis

Sample sizes for patch-clamp experiments on HEK293T cells were based on earlier work from our team and literature, with a minimal sample size of n = 5. Sample sizes for calcium imaging on HEK293T cells and DRG neurons was based on the literature but always included a minimum of 5 independent experiments containing >100 cells per experiment. The sample sizes for all the in vivo experiments was based on a power analysis using statistical data from pilot experiments, aimed at detecting a difference of at least 20% between groups with an α of 0.05 and a power of 0.8. Data analysis, statistics, and data display were performed using Origin 9.1 (OriginLab). All data are presented as the mean ± SEM from n biological replicates. Normality was tested using the Shapiro–Wilk test. The specific statistical tests used to determine the significance of the differences between experimental data sets are indicated in the figure legends. *P* values of <0.05 were considered significantly different.

3. Results

3.1. Effect of oxaliplatin on the TRPM3 channel activity

First, we tested whether oxaliplatin has a direct agonistic effect on the TRPM3 activity using microfluorimetric calcium imaging in HEK-mTRPM3 and HEK-hTRPM3 cell lines (Supplementary Fig. 1, available at http://links.lww.com/PAIN/B806). Direct application of oxaliplatin (100 μM) did not evoke any response in the intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, whereas stimulation with the TRPM3 agonist, PS (40 μM), induced a strong TRPM3-mediated Ca^{2+} influx. Similar results were obtained in isolated DRG neurons from mice (Supplementary Fig. 1, available at http://links.lww.com/PAIN/B806). In addition, to test the effect of oxaliplatin on the TRPM3 activity, HEK-mTRPM3 and HEK-hTRPM3 cell lines and DRG neurons were first stimulated by PS and followed by coapplication of PS + oxaliplatin. In all different expression systems, coapplication of PS + oxaliplatin was without effect on the PS-induced Ca^{2+} influx, suggesting that oxaliplatin has no direct effect on the TRPM3 channel activity

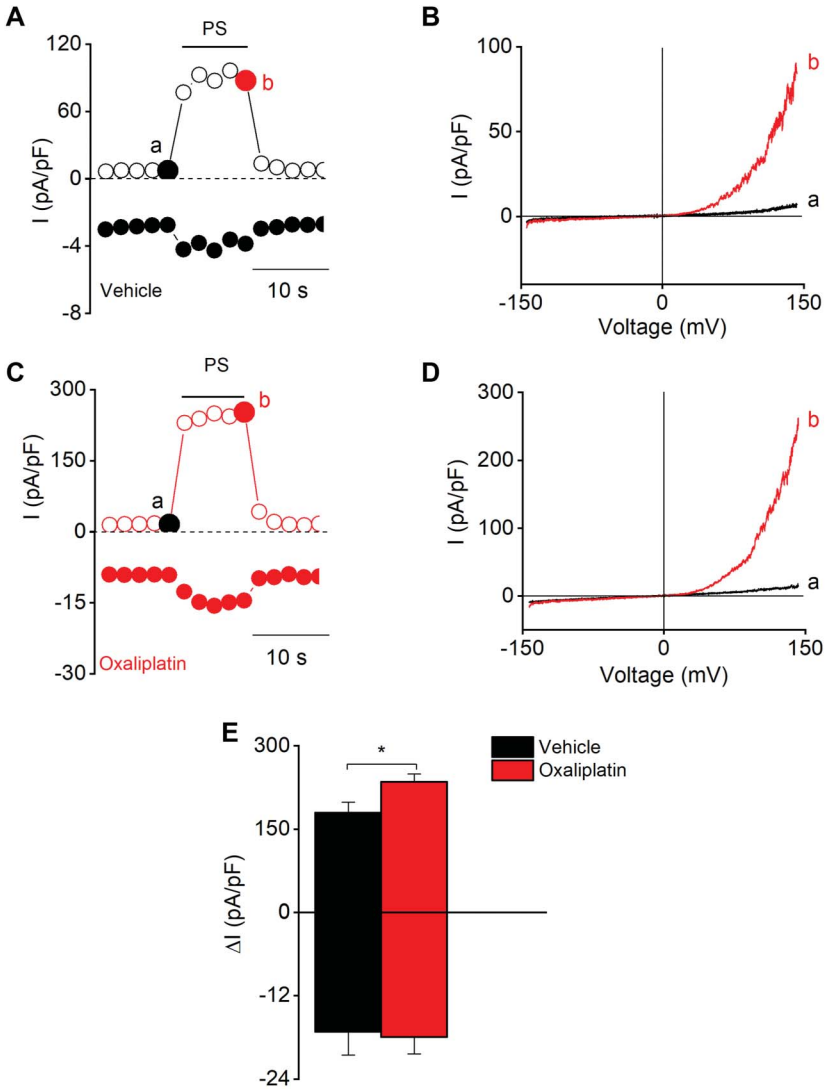

Figure 2. Modulation of TRPM3 function in HEK293-mTRPM3. (A) Time course at ± 150 mV of a whole-cell patch-clamp recording showing the effect of pregnenolone sulphate (PS; 40 μM) on HEK-mTRPM3 expressing cells after vehicle preincubation (n = 16). (B) Current (I)−voltage (V) relationship corresponding to the time points indicated in A. (C) Time course at ± 150 mV of a whole-cell patch-clamp recording showing the effect of PS (40 μM) on HEK-mTRPM3 expressing cells after 24 hours of oxaliplatin (100 μM) preincubation (n = 16). (D) I-V relationship corresponding to the time points indicated in C. (E) Mean current increase at ± 150 mV in HEK-TRPM3 cells on PS application after 24 hours of vehicle or oxaliplatin preincubation. Where *$P < 0.05$ (Mann–Whitney *U* test). PS, pregnenolone sulfate.

(Supplementary Fig. 2, available at http://links.lww.com/PAIN/B806). Next, we investigated the effect of long-term incubation with oxaliplatin (100 μM for a period of 24 hours) on TRPM3 expression and activity, in HEK-mTRPM3 and HEK-hTRPM3 cell lines and in DRG sensory neurons. In transiently transfected HEK293T cells with murine TRPM3, we measured significantly increased basal calcium levels at 37°C in cells treated with oxaliplatin compared with vehicle-treated cells (**Fig. 1A**); the increased cytosolic calcium levels could be partially reversed by the application of the TRPM3 inhibitor primidone (100 μM) (**Figs. 1A and B**).[12] Moreover, $[Ca^{2+}]_i$ responses to PS or heat were significantly larger in oxaliplatin preincubated cells compared with vehicle-treated cells (**Figs. 1C–F**). The heat-induced $[Ca^{2+}]_i$ influx was fully inhibited in the presence of the TRPM3 antagonist isosakuranetin (20 μM), which further validates the heat induced calcium response by the activation of TRPM3 (Supplementary Fig. 3, available at http://links.lww.com/PAIN/B806). Similar results were obtained using the human TRPM3 isoform (Supplementary Fig. 4, available at http://links.lww.com/PAIN/B806). These observations were further validated by whole-cell patch-clamp experiments, showing larger PS-induced current amplitudes in oxaliplatin pretreated HEK-mTRPM3 cells compared with vehicle controls (**Fig. 2**). Next, we evaluated whether oxaliplatin modifies the opening of the noncanonical pore of TRPM3.[36] Therefore, cells were first stimulated with clotrimazole (Clt) and afterwards stimulated with coapplication of PS + Clt. No differences were observed in the amplitude of the noncanonical current at −150 mV between oxaliplatin and vehicle-treated cells (Supplementary Fig. 5, available at http://links.lww.com/PAIN/B806).

We also tested the effect of oxaliplatin pretreatment on the PS responses in primary cultures of DRG neurons derived from wild-type mice. Application of PS (40 μM) to neurons evoked a $[Ca^{2+}]_i$ response that was significantly larger in oxaliplatin-pretreated cells compared with vehicle controls (**Figs. 3A and B**). Moreover, the percentage of PS-responsive DRG neurons was increased after oxaliplatin treatment compared with vehicle controls (**Fig. 3D**). Interestingly, similar results were obtained when comparing DRG neurons isolated from mice at day +1 after oxaliplatin treatment (6 mg/kg) compared with vehicle (5% glucose)-treated mice (**Figs. 3E, F, H**). To validate the neuronal character of the cells, a high K^+ (50 mM) solution was applied at the end of the experiment to induce a depolarization of the membrane potential and to activate voltage-dependent Ca^{2+} channels (Ca_V). Intriguingly, the amplitude of the $[Ca^{2+}]_i$ influx induced by the high K^+ solution was significantly increased in DRG neurons preincubated with oxaliplatin (**Figs. 3C and G**). These results can be explained by the upregulation of the protein levels of voltage-gated calcium channels after 24 hours incubation with oxaliplatin.[23] Note that this in vivo treatment of the mice with a single dose of oxaliplatin had no effect on electrophysiological parameters of sensory nerve conduction (Supplementary Fig. 6, available at http://links.lww.com/PAIN/B806), indicating that the integrity of the sensory nerves was preserved.

To elucidate whether the increased TRPM3 functionality was a result of increased mRNA expression, RT-qPCR was performed on DRG neurons isolated from mice 24 hours after oxaliplatin or vehicle injection. This analysis did not reveal any significant change in TRPM3-encoding mRNA, but confirmed increased mRNA expression for TRPM8, in line with earlier work.[10] A similar outcome was also observed on HEK-mTRPM3 after 24 hours of

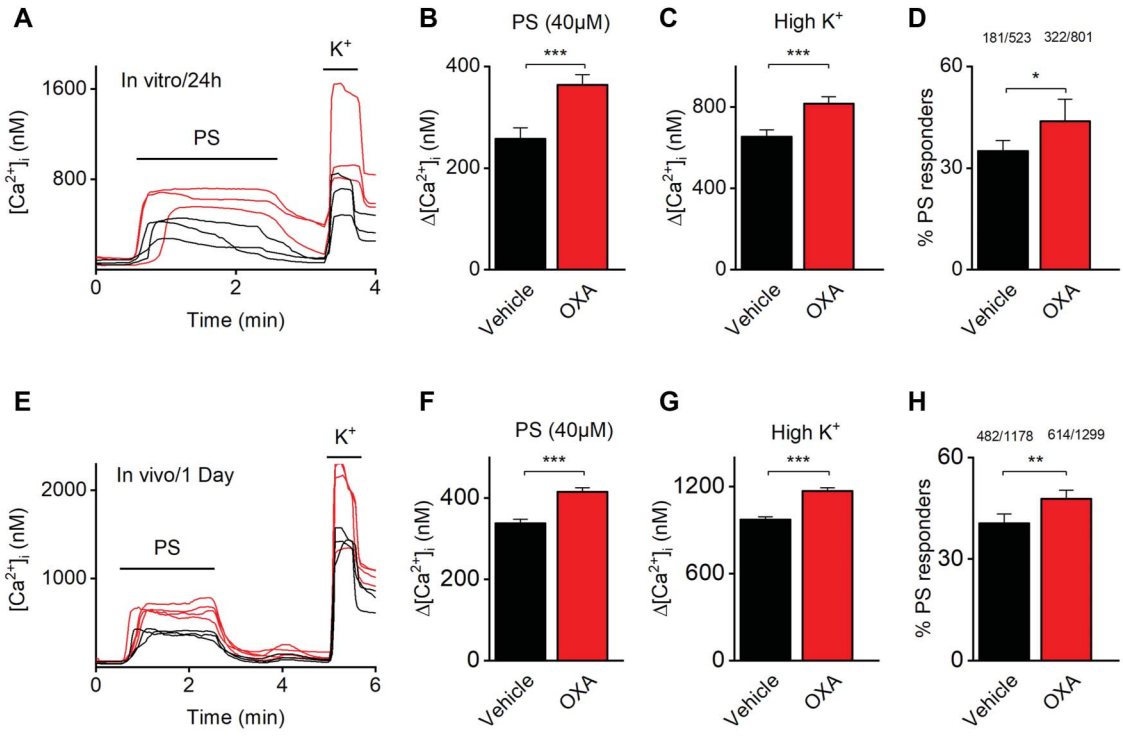

Figure 3. Modulation of TRPM3 function in DRG sensory neurons after oxaliplatin pretreatment. Time course of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (mean ± SEM) in DRG isolated from wild-type animals $TRPM3^{+/+}$ in response to PS (40 μM) and high K^+ (50 mM) after oxaliplatin (100 μM; red) and vehicle treatment in black (A) and after 24 hours after oxaliplatin (6 mg/kg; red color) or vehicle i.p injection (black color) (E). (B, C, F, G) Quantification of calcium responses for experiments as in panel A and E, respectively, *** $P < 0.001$ (Mann–Whitney U test). Percentage of sensory neurons derived from $TRPM3^{+/+}$ responding stimulation by PS (40 μM) after oxaliplatin and vehicle preincubation (D) and after 24 hours after oxaliplatin (6 mg/kg) or vehicle i.p injection (H). Where *$P < 0.05$ (χ^2 test); **$P < 0.01$ (χ^2 test). DRG, dorsal root ganglia; PS, pregnenolone sulfate.

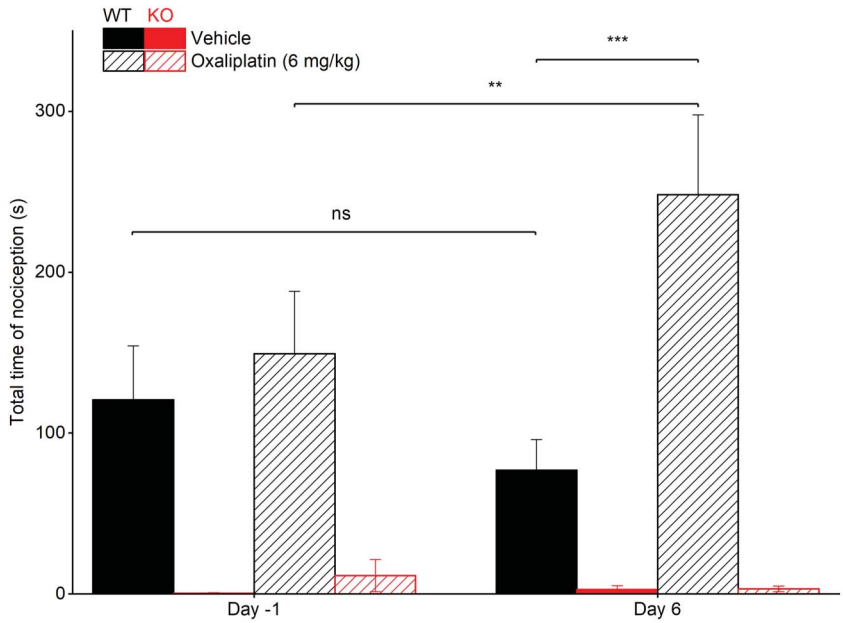

Figure 4. Nocifensive responses to PS after oxaliplatin and vehicle injection. (A) Total duration of nocifensive behavior (paw licks and lifts during a period of 10 minutes) in response to intraplantar injection of pregnenolone sulfate (PS, 5 nmol/paw) in TRPM3$^{+/+}$ and TRPM3$^{-/-}$ mice (n = 8 animals/genotype). Data are represented as mean ± SEM. Statistically significant changes in the duration of PS-evoked pain responses were assessed using the 2-way ANOVA repeated measurement statistical test with Sidak-holm post hoc test. Where $^{**}P < 0.01$; $^{***}P < 0.001$. ANOVA, analysis of variance; WT, TRPM3$^{+/+}$; KO, TRPM3$^{-/-}$.

oxaliplatin preincubation. (Supplementary Fig. 7, available at http://links.lww.com/PAIN/B806).

3.2. Oxaliplatin modulates TRPM3-dependent pain responses in vivo

To investigate whether oxaliplatin-induced peripheral neuropathic pain is associated with alterations in TRPM3-dependent pain responses, we compared the in vivo TRPM3 function in oxaliplatin-treated and vehicle-treated mice. To this aim, animals were submitted to a TRPM3-specific chemogenic pain model consisting of the intraplantar injection of PS (20 μL, 5 nmol).[35] The nociceptive response was quantified as the total time of nociception, ie, the time during which the animals licked, lifted, or guarded the affected paw. Before oxaliplatin treatment, the intraplantar injection of PS-evoked robust nocifensive behavior in wild-type mice, which was not observed in TRPM3-deficient mice, similar to earlier studies.[35] Interestingly, the PS-induced pain response in wild-type mice was significantly enhanced at day 6 after injection with oxaliplatin (6 mg/kg) compared with vehicle-treated mice or to the pretreatment condition. Even after oxaliplatin treatment, TRPM3-deficient mice remained nonresponsive to PS, confirming the TRPM3 specificity of the

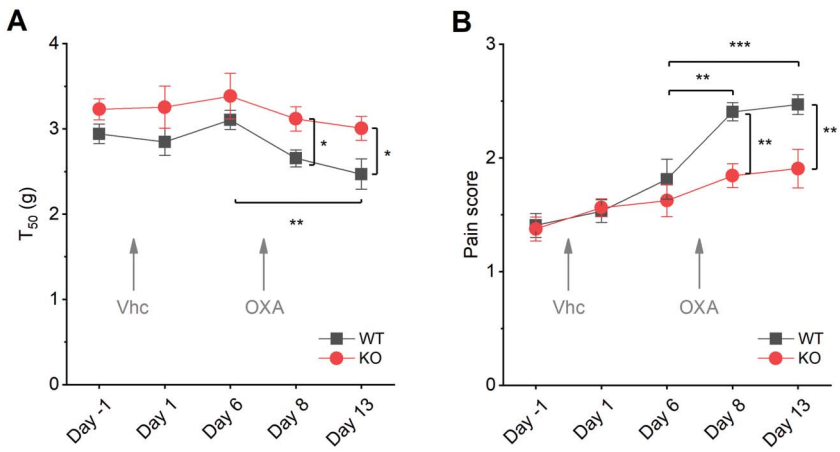

Figure 5. TRPM3 genetic ablation reduces oxaliplatin-induced mechanical allodynia. (A and B) A single intraperitoneal injection of oxaliplatin (6 mg/kg) induces in TRPM3$^{+/+}$ mice (black color) a time-dependent reduction in mechanical nociceptive threshold and increase in cold sensitivity, respectively. Mechanosensitivity (A) and cold sensitivity (B) were measured at baseline (Day −1), 24 hours after the injection of vehicle (Day 0) and after 6 days (Day 6). On day 7 (Day 7), animals treated with oxaliplatin and mechano (A) or cold sensitivity (B) were measured after 24 hours (Day 8) and after 6 days (Day 13) after injection. The development of mechanical and cold allodynia observed in TRPM3$^{+/+}$ animals (black) after oxaliplatin treatment was decreased in TRPM3$^{-/-}$ mice (red color). Data are presented as mean ± SEM (n = 8 mice per group). Statistically significant changes were assessed by using the 2-way ANOVA repeated measurement statistical test with the Sidak-Holm post hoc test. Where $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. ANOVA, analysis of variance; Vhc, vehicle; OXA, oxaliplatin; WT, wild-type; KO, TRPM3$^{-/-}$.

assay (**Fig. 4**). Taken together, these data indicate that TRPM3 is functionally upregulated in DRG neurons after oxaliplatin treatment.

3.3. Involvement of TRPM3 in oxaliplatin-induced mechanical allodynia and cold hypersensitivity

To evaluate whether TRPM3 is involved in oxaliplatin-induced cold hypersensitivity and mechanical allodynia, we tested the effect of a single administration of oxaliplatin on behavioral responses to mechanical and cold stimuli in control and TRPM3$^{-/-}$ mice (acute oxaliplatin neuropathy). In wild-type animals, the withdrawal threshold to a mechanical stimulus was significantly reduced 6 days after oxaliplatin treatment (Day 13) compared with the pretreatment levels (Day 6) (**Fig. 5A**). Notably, oxaliplatin-induced mechanical hypersensitivity was significantly suppressed in TRPM3$^{-/-}$ mice (**Fig. 5A**). Similarly, wild-type mice exhibited significant and robust cold hypersensitivity in the acetone spray test at day 8 and day 13 after oxaliplatin treatment (Day 7), whereas TRPM3$^{-/-}$ mice did not develop such cold hypersensitivity (**Fig. 5B**).

Increased activity of DRG neurons is associated with higher levels of the phosphorylated form of the pronociceptive pERK, which has been observed in animal models of inflammatory and neuropathic pain, including oxaliplatin-induced neuropathy.[14,16] We, therefore, compared pERK expression levels analysed by immunohistochemical stainings of DRG neurons isolated from wild-type and TRPM3$^{-/-}$ mice 24 hours after dosing of oxaliplatin

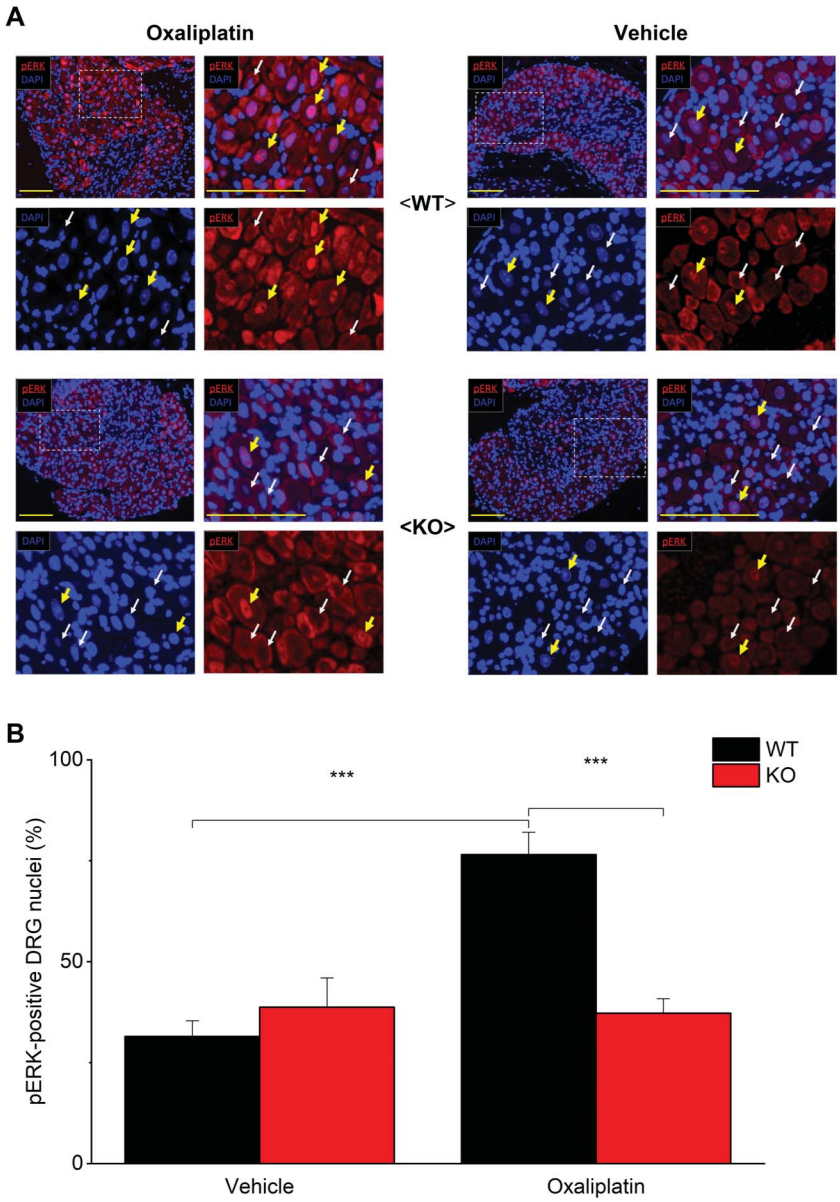

Figure 6. pERK expression levels in DRG of wild-type and TRPM3$^{-/-}$ mice after dosing of oxaliplatin. (A) Sections of pERK immunostaining in DRG neurons of TRPM3-WT and TRPM3-KO mice at 24 hours after oxaliplatin or vehicle injection. Scale bars, 50 μm. (B) The percentage of pERK-positive cells in the DRG sections was counted (n = 6 sections, from 3 independent DRG preparations). Data are expressed as the mean ± SEM. Statistically significant changes were assessed by using the 2-way ANOVA statistical test with the Sidak-Holm post hoc test. Where ***P <0.001. ANOVA, analysis of variance; DRG, dorsal root ganglia; pERK, protein ERK; WT, wild-type; KO, TRPM3$^{-/-}$.

(6 mg/kg) or vehicle. We observed that the number of pERK-positive cells was significantly increased in DRG cell bodies of oxaliplatin-injected wild-type animals compared with vehicle controls animals. Importantly, oxaliplatin did not cause any increase in pERK expression in DRG neurons derived from TRPM3$^{-/-}$ mice (**Fig. 6**). Taken together, these data indicate that TRPM3 deficiency protects mice from oxaliplatin-induced mechanical allodynia and cold hypersensitivity and prevents the oxaliplatin-induced upregulation of the pronociceptive pERK.

3.4. Effects of TRPM3 inhibition on the development of chemotherapy-induced peripheral neuropathic pain

To investigate whether acute pharmacological inhibition of TRPM3 affects CIPNP, the effect of the TRPM3 antagonist isosakuranetin[26] was tested on oxaliplatin-induced cold and mechanical hypersensitivity (**Fig. 7**), using the opioid tramadol (5 mg/kg) as a positive control.[18] In these experiments, the cold and mechanical sensitivity of wild-type mice was tested at baseline (Day −1) and at day 6 after a single dose of oxaliplatin was injected, confirming significant hypersensitivity similar to the experiments shown in **Figure 5**. After the first testing on day 6, animals received a single i.p. injection with isosakuranetin (2 mg/kg), tramadol, or vehicle, followed by repeat testing for cold and mechanical sensitivity. In comparison with vehicle, both isosakuranetin and tramadol resulted in a significant increase in mechanical threshold and decrease in the cold nociceptive response (**Fig. 7**). Taken together, these findings indicate that TRPM3 antagonism alleviates single-dose oxaliplatin-induced cold and mechanical hypersensitivity.

4. Discussion

Oxaliplatin as a drug has been used for a long time in the treatment and management of cancer. Despite the effectiveness of oxaliplatin, the drug comes with substantial side effects, including hair loss, diarrhea, mouth sores, change in taste, dizziness, nosebleeds, and painful peripheral neuropathy. Several mechanisms have already been proposed in the literature to explain the dose-limiting painful side effects often linked with the administration of chemotherapeutics agents such us oxaliplatin. For instance, oxaliplatin reportedly induces modifications of several intracellular signaling pathways[9] and alters activity of voltage-gated sodium and calcium channels and the sensory TRP channels TRPA1, TRPV1, and TRPM8.[6,20,23,37] Recently, another member of the TRP channel superfamily, TRPM3, has increasingly received attention as a potential key player in acute inflammatory and neuropathic pain signaling and as a potential target for new analgesic treatments.[27] However, the contribution of TRPM3 to CIPNP remained unstudied. Here, we present evidence that TRPM3 is critically involved in the development of acute oxaliplatin-induced neuropathic pain and demonstrate that both genetic ablation and pharmacological inhibition of the channel significantly reverts oxaliplatin-induced mechanical hyperalgesia and cold hypersensitivity.

Oxaliplatin treatment was associated with increased TRPM3 activity, which we demonstrated in heterologous expression systems, in isolated sensory neurons, as well as and in an in vivo chemogenic pain model. The mechanisms whereby oxaliplatin affects TRPM3 activity currently remain unknown. In contrast to earlier findings in inflammatory pain models,[17,34,39] we did not find any evidence for increased TRPM3 expression at the mRNA level after oxaliplatin treatment. However, we confirmed increased mRNA levels for the cold-activated TRPM8, which is in line with earlier studies.[3,7] These data suggest that oxaliplatin enhances TRPM3 activity at the posttranscriptional level. Based on our experiments, we can exclude a direct agonistic effect of oxaliplatin on TRPM3 channel function or the noncanonical TRPM3 pore suggesting that the enhanced channel activity is the consequence of increased protein expression at the plasma membrane or sensitized channel gating. In this respect, studies on other ion channels, including TRPV1 and TRPA1, point towards an important role for reactive oxygen species (ROS), which are generated after oxaliplatin-induced mitochondrial dysfunction[22] and can affect the activity of these channels through posttranslational modifications to cysteine or proline residues. Further research is needed to test whether similar ROS modulation occurs at TRPM3.

Oxaliplatin treatment in cancer patients frequently causes neuropathic pain, which encompasses cold hypersensitivity and mechanical allodynia. These symptoms develop within the first hours to days after dosing and can reproducibly be mimicked in rodent models. Here, we demonstrate a key role for TRPM3 in the development of these acute symptoms in mice. First, we show that the hypersensitivity towards cold and mechanical stimuli, which develops after single dose injection of oxaliplatin in wild-type mice, is suppressed in TRPM3$^{-/-}$ mice. These findings establish that TRPM3 is required for the development of the

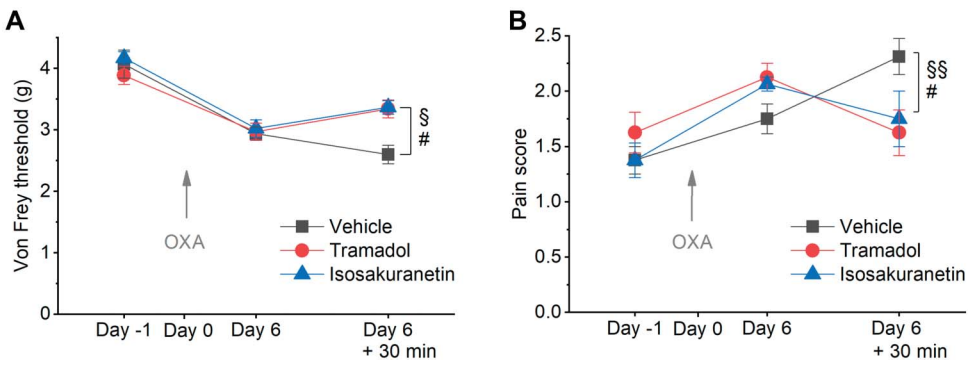

Figure 7. Pharmacological inhibition of TRPM3 reduces neuropathic pain induced by oxaliplatin. The effect is tested in the electronic Von Frey assay and acetone spray test at day 6 after oxaliplatin treatment. (A and B) Effect of isosakuranetin (2 mg/kg) and tramadol (5 mg/kg) as positive control and vehicle used in the intervention protocol on nociceptive mechanical threshold and escape behavior score in mice treated with oxaliplatin. Results are shown as mean ± SEM of n = 8 mice per condition. Statistical analysis: 2-way ANOVA repeated measurements with the Sidak-holm post hoc test. Where §§ Vhc vs tramadol (P <0.01); ## Vhc vs isosakuranetin (P <0.01); # Vhc vs isosakuranetin (P <0.05). ANOVA, analysis of variance.

hallmark symptoms of acute oxaliplatin-induced neuropathic pain. Moreover, we observed that the oxaliplatin-induced upregulation of pERK in the cell bodies of DRG neurons, which is a biochemical marker of increased neuronal activation, is absent in TRPM3$^{-/-}$ mice. In addition, we found that the pharmacological inhibition of TRPM3 using isosakuranetin could effectively inhibit oxaliplatin-induced mechanical and cold hypersensitivity, with a similar efficacy as the opioid tramadol. The analgesic effect of isosakuranetin was not observed in TRPM3$^{-/-}$ mice, suggesting for an on-target effect of isosakuranetin on TRPM3. Taken together, these findings reveal TRPM3 as a promising potential new target to treat painful hyperesthesia associated with oxaliplatin treatment.

Our observations that genetic ablation or pharmacological inhibition of TRPM3 has a significant effect on oxaliplatin-induced hypersensitivity to cold and mechanical stimuli may seem at odds with the known activation modalities of TRPM3 as a sensory ion channel. Indeed, TRPM3 is primarily known to be activated by and involved in the sensation of heat[32,35] and specific chemical ligands; as such, cold temperatures reduce the TRPM3 channel activity, and there is limited experimental evidence to link TRPM3 to the detection of mechanical stimuli. One possible explanation for our findings could be that the oxaliplatin-induced increased functionality of TRPM3, a depolarizing cation current, increases the excitability of the large set of TRPM3-expressing DRG neurons, including those involved in detecting cold and mechanical stimuli. In such a scenario, inhibition of TRPM3 function would revert the sensory neuronal hyperexcitability, thereby reducing the thermal and mechanical sensitivity to control levels. A similar role for TRPM3 in modulating nociceptor excitability has also been proposed in a mouse model of inflammatory hyperalgesia.[26] In those experiments, inhibition of TRPM3 in neurons innervating inflamed tissue reduced the responses to agonists of TRPA1 and TRPV1, which are broadly coexpressed with TRPM3 in nociceptor neurons.

In conclusion, we have demonstrated that the chemotherapeutic drug oxaliplatin evokes enhanced TRPM3 activity in vitro and in vivo and is essential for the development of oxaliplatin-induced cold and mechanical hypersensitivity. TRPM3, thus, represents a potential new target for the treatment of neuropathic pain in patients undergoing chemotherapy.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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