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Activation of KCNQ channels prevents paclitaxel-induced peripheral neuropathy and associated neuropathic pain

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Abstract

Paclitaxel-induced peripheral neuropathy (PIPN) and associated neuropathic pain are the most common and serious adverse effects experienced by cancer patients receiving paclitaxel treatment. These effects adversely impact daily activities and consequently the quality of life, sometimes forcing the suspension of treatment and negatively influencing survival. Patients are usually at high risk of developing PIPN if paclitaxel induces acute pain, which strongly suggests that an acute increase in the excitability of nociceptors underlies the chronic alterations of PIPN. KCNQ/Kv7 channels are widely expressed in the primary sensory neurons to modulate their excitability. In the present study, we show that targeting KCNQ/Kv7 channels at an early stage is an effective strategy to attenuate the development of PIPN. We found that paclitaxel did not decrease the expression level of KCNQ/Kv7 channels in the primary sensory neurons as detected by qRT-PCR and Western blotting. However, retigabine, which is a specific KCNQ/Kv7 channel opener, significantly attenuated the development of PIPN, as shown by both morphologic and behavioral evidence. We also observed that retigabine had no obvious effect on the chemosensitivity of breast cancer cells to paclitaxel. While retigabine has been approved by the FDA as an anticonvulsant, our study suggests that this drug can be repurposed to attenuate the development of PIPN.

Perspective—Paclitaxel-induced peripheral neuropathy and associated neuropathic pain are severe and resistant to intervention. The results of our study demonstrated that retigabine (a clinically available medicine) can be used to attenuate the development of paclitaxel-induced peripheral neuropathy.

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Keywords

K^+ channels; retigabine; hyperexcitability; paclitaxel; neuropathy; pain; prevention

1. Introduction

Paclitaxel is a commonly used chemotherapeutic agent for the treatment of breast and other cancers [12]. Unfortunately, its use is often associated with significant neurotoxicity, especially resulting in sensory peripheral neuropathies that are often accompanied by neuropathic pain and are frequently severe and resistant to intervention. Paclitaxel-induced peripheral neuropathy (PIPN) results from damage to, or dysfunction of, peripheral nerves, including sensory, autonomic, and motor neurons. The incidence and severity of PIPN is duration- and dose-related, such that more than 60% of patients receiving paclitaxel-based chemotherapy experience peripheral neuropathy in first 3 months [58]. However, neither analgesic medicine nor neuroprotective drugs yield promising effects on PIPN [19]. Thus, the use of paclitaxel for cancer patients can be limited by unmanageable peripheral neuropathy, resulting in premature suspension of treatment and decreased survival.

PIPN is both persistent and refractory to therapy once it becomes chronic. Thus, preventing its development rather than palliation would be the best strategy. Recent studies indicate that paclitaxel induces inward currents and directly excites human dorsal root ganglion (DRG) neurons *in vitro* [39, 40]. Paclitaxel also induces an immediate response of visceral and peripheral nociception in rodents [56]. It is noteworthy that patients are usually at high risk of developing PIPN if paclitaxel induces acute pain [42, 53], which suggests that an acute increase in the excitability of nociceptors underlies the chronic alterations of PIPN. Thus, keeping neurons at rest or in a hyperpolarized state during paclitaxel administration could be a workable strategy to prevent/attenuate the development of PIPN and associated neuropathic pain.

Decreasing the hyperexcitability of peripheral neurons by calcium and magnesium infusions has been proposed as a strategy to prevent oxaliplatin-induced peripheral neuropathy [3, 23]. However, this treatment has recently been evaluated as ineffective [27]. An alternative therapeutic approach is to target K^+ channels, which have an essential role in setting the resting membrane potential and thus controlling the excitability of neurons, including nociceptors. KCNQ channels (also called M channels) constitute the Kv7 subfamily (Kv7.1-Kv7.5) [32, 60]. Many neurons in rat DRGs, including small neurons that respond to capsaicin (presumptive nociceptors), express Kv7.2, Kv7.3 and/or Kv7.5 [49, 55]. Agents that promote the opening of KCNQ/Kv7 channels have been shown to inhibit behavioral hypersensitivity in both inflammatory and other neuropathic pain models [5, 41, 49, 57, 65], and to provide some neuroprotection [6, 54]. Notably, retigabine, a KCNQ/Kv7 channel opener [43], is already used clinically as an anticonvulsant [14]. We thus tested whether early, brief application of retigabine will prevent the development of PIPN. Here we report that early activation of KCNQ channels with retigabine suppresses the degeneration of peripheral nerves and effectively reduces signs of chronic pain.

2. Materials and methods

All procedures conformed to the guidelines of the International Association for the Study of Pain, and were approved by the animal care and use committee of the University of Texas Medical Branch at Galveston. Male, adult, Sprague-Dawley rats (200–300g) were used in this study. Animals were housed 2 per wood chips bedding cage in a controlled environment (12 hour reversed light/dark cycle, 21 ± 1°C) with standard food and water. Cages were changed twice per week. The rats were allowed to adjust to their environment for a week before the experiment.

2.1. Administration of Paclitaxel and retigabine

The rats (200–300g) received intraperitoneal (i.p.) injections of clinically formulated paclitaxel (TEVA Pharmaceutical Inc, North Wales, PA) at a dosage of 2 mg/kg in 0.9% saline every other day for a total of four injections (Days 1, 3, 5, and 7, 8:00 to 10:00 am) [69]. In order to match the clinical formulation of paclitaxel, the vehicle stock solution was made of equal parts of cremophor EL (Sigma) and Ethanol. Sham animals received an equivalent volume of the vehicle in 0.9% saline. The volume of paclitaxel and vehicle were injected according to their weight, i.e., a 250 g rat received 0.5 ml injection. For all of the experiments, rats were randomly divided into 3 groups: 1) Sham group – Rats received vehicle of paclitaxel and vehicle of retigabine (saline) in an identical schedule and volume as groups 2 and 3. 2) Paclitaxel plus retigabine – From day 1 to 10, paclitaxel-treated animals were administrated with retigabine (10 mg/kg, i.p.; Torondo Research Chemicals, Canada) twice daily. 3) Paclitaxel + vehicle group – From day 1 to 10, paclitaxel-treated animals received saline (vehicle of retigabine, i.p.) twice daily. On day 1, 3, 5, and 7, retigabine and its vehicle of morning session were injected 30 min prior to paclitaxel injection. The second injection of retigabine and its vehicle was performed at late afternoon.

2.2. Behavioral tests

All behavioral tests were performed in the active (dark) phase. Animals received tests for hindlimb reflex sensitivity prior to injection of paclitaxel (baseline) with or without retigabine treatment, and posttests on days 12, 28, and 35 after paclitaxel. All behavioral data were collected by staff blinded to any drug treatment during the animals' active phase under red light. Prior to each test, the animal was habituated for 20 min per day in each of the testing chambers. Signs of mechanical sensitivity were tested with a series of calibrated von Frey filaments (Stoelting, Wood Dale, IL) delivered to the glabrous surface of the hindpaws. Thresholds were determined with the "up-down" method [11]. Only one test series was applied to each hindpaw. Conditioned place preference (CPP) tests were performed as previously described [61, 66]. Briefly, each rat was habituated to a CPP device (Med Associates Inc, VT) on the first day following a 3-day sequence of twice daily conditioning sessions (conditioning phase). In one session, the rat was restricted to the innately non-preferred white chamber for 60 min, starting 5 min after delivery of retigabine (10 mg/kg, i.p.; as an anesthetic for CPP test). In another daily session, the rat was placed for 60 min in the innately preferred black chamber 5 min after delivery (i.p.) of saline (vehicle of retigabine). In the testing phase, 1 day after the last conditioning session, the rat was placed (without any injection) in the open central gray chamber, and the time spent in each of the 3 chambers

was recorded for 15 min. Conditioning to retigabine was indicated by greater time spent in the white compared to the black chamber during the testing phase.

2.3. Immunohistochemistry

After finishing all behavioral tests (day 28), a 3-mm glabrous skin section from the hindpaw was removed under deep anesthesia induced by injection of 75 mg/kg Beuthanasia (Merck Animal Health, Kenilworth, NJ) and fixed in Zamboni's fixative overnight at 4 degrees. Tissues were transferred to 20% sucrose for at least 24 hours and frozen in optimal cutting temperature (OCT) compound. Tissues were then cut into 25 μm transverse sections. Sections were directly mounted onto gel-subbed glass slides, rinsed 3X in tris-buffered saline (TBS), and blocked with 5% serum for 1 hour. Sections were incubated with a combination of primary antibodies against PGP9.5 (Proteintech Group Inc, IL; Cat#17430) and collagen IV (Southernbiotech, AL; Cat# 1340) and then secondary antibodies. After rinsing in TBS, sections were mounted with coverslips with Vectashield (Vector Labs). Negative controls omitted primary antibodies. Labeled sections were examined with a Nikon Eclipse 600 (Nikon Instruments Inc, Melville, NY, USA) fluorescence microscope. The length of the epidermis within a field of view was measured using Image J software (NIH).

2.4. Quantification of intraepidermal nerve fibers

The intraepidermal nerve fibers were quantified as previously reported [7]. Briefly, 4–5 slices from each animal and three fields of view from each slice were randomly selected. PGP9.5-stained nerve fibers that crossed the dermal/epidermal junction (stained with collagen IV) were counted, and the length of the epidermis within each field of view was measured. IENF density was calculated as the total number of fibers per unit length of epidermis (IENF/mm). Tissues were collected and marked by another technical staff member and treatment information was revealed only after IENF quantification.

2.5. Quantitative RT-PCR analysis

Total RNA was extracted from homogenized DRGs dissociated 12 days after initial paclitaxel treatment with on-column DNase digestion (E.Z.N.A. Total RNA Kit I) and cDNA was synthesized by MMLV reverse transcriptase (Invitrogen) using random primers. Rat KCNQ2 primers were 5'-CCGGCAGAACTCAGAAGAAG-3' (forward) and 5'-TTTGAGGCCAGGGGTAAGAT-3' (reverse) [55]; rat KCNQ3 primers were 5'-ATACACATTTATCTGCTCTTCCTTTTA-3' (forward) and 5'-TGCTCTCAGTTTATCCGAATCAA-3' (reverse) [10], and rat GAPDH primers were 5'-CCCCCAATGTATCCGTTGTG-3' (forward) and 5'-TAGCCCAGGATGCCCTTTAGT-3' (reverse) [52]; rat KCNQ5 primers were 5'-CCTGGCGTACACGAGAGT-3' (forward) and 5'-TTTGACTGGGCGAACTGA-3' (reverse). Abundance of mRNA was measured by real-time PCR (LightCycler 480; Roche) with SYBR Green Master Mix (Sigma). Preincubation at 95°C for 3 min was followed by 45 amplification cycles (95°C for 30 s, 57°C for 30 s, and 72°C for 30 s) and fluorescence collection at 60°C. Gene expression was normalized to GAPDH and averaged over three replicates from each of five animals in each group. Two independent experiments were performed.

2.6. Western Blotting

After finishing all behavioral tests, animals were deeply anesthetized with Beuthanasia and perfused with ice-cold PBS. The L4 and L5 of the spinal cords and dorsal root ganglions from each rat were removed and immediately placed in a 1.5 ml Eppendorf tubes on dry ice. Tissues were homogenized in 500 μl of lysis buffer (RIPA, Teknova) containing a protease inhibitor cocktail (Sigma). After homogenization, samples were sonicated 3 times (10 s pulses), and centrifuged at 14,000 rpm for 10 min at 4 °C. The protein concentration of lysates was determined by the BCA assay (Pierce BCA Protein Assay Kit). Samples were prepared for SDS-PAGE (Bio-Rad, 4–20% Tris-HCl) by 1:1 dilution with Laemmli sample buffer, and 30 μg of protein was loaded in each well. After electrophoresis, the gel was transferred to a PVDF membrane and blocked with 10% nonfat dry milk in PBS + 0.1% Tween 20 prior to incubation with antibody against GFAP (Millipore, USA; AB5541), KCNQ5 (Alomone Labs, Israel; APC-155), Iba-1 (Wako, 019–19741), and β-actin (Abcam, MA; ab8226) overnight at 4° C. The membrane was incubated with HRP-conjugated anti-rabbit or anti-mouse IgG (Jackson ImmnuoResearch, PA) for 1hour at room temperature and developed using the ECL kit (Pierce). Protein expression was quantified by optical density using Image J software (NIH). Color molecular weight standards were run on each gel and β-actin was detected as a loading control.

2.7. Electron microscopy and analysis of mitochondria

Analysis of axon mitochondria by electron microscopy was performed as previously described [21]. Briefly, the tibial nerves (0.5 cm) excised from the lower leg of the rats were post-fixed for 2 hours in fixative containing 1% glutaraldehyde and 1% paraformaldehyde in 0.1 M PB (pH 7.4), followed by incubation in 10% sucrose at 4° C for 12 hours. Tissues were then incubated in 1% osmium tetroxide, dehydrated in ascending concentrations of alcohol and propylene oxide at room temperature, and embedded in Epon-Araldite. Ultrathin cross-sections of 70 nm were prepared with a microtome using a diamond knife, collected onto Formvar-coated grids, and counterstained. Four ultrathin cross-sections from each animal were randomly selected and sampled to take electron photomicrographs with a JEOL JEM-1400 transmission electron microscope (Jeol, USA) at three magnifications of X800, X3000 and X40000. Electron photomicrographs that were taken at X3000 magnification help us to identify A-fibers, C-fibers and Remak bundles, as photomicrographs taken at X40000 magnification were used to determine normal and abnormal morphology of mitochondria. Mitochondria in axons (A-fibers and C-fibers) were viewed and photographed randomly by same investigator blinded to treatment conditions. As previously reported [21], mitochondria in the axoplasm of each axon and in the cytoplasm of each myelinating and non-myelinating Schwann cell were counted. Normal mitochondria had at least one axis of at least 165 nm in length with intact double membranes. Mitochondria were counted as atypical if they had at least a two-fold increase in diameter and/or if 50% or more of the interior was electron-lucent. Nerve fibers containing one or more atypical mitochondria were counted.

2.8. Tumor cytotoxicity assay

SKBR3 cells (ACCT, VA) were seeded at a density of 2.5×10^4 cells/cm^2 onto 24 well plates for experiments on the following day. The cell viability was assessed by measuring mitochondrial activity in reducing MTT (Thiazolyl blue tetrazolium bromide, Fisher scientific, 158990050) to formazan. After exposure to paclitaxel (0, 1, 5, 10, 20, 40, 60, 80, and 100 nM) with and without retigabine (10 μM) for 24, 48, or 72 hours, cells were washed with Dulbecco's phosphate-buffered saline (DPBS), then 500 μl of medium and 50 μl of MTT assay solution (5 mg/ml) were added and incubated at 37°C in 5% CO_2 for 4 hours. The incubation medium was carefully removed and re-suspend formazan in 500 μL DMSO. The amount of formazan dye was measured from the absorbance at 700 nm with a reference wavelength of 620 nm using a microplate reader.

2.9. Data Analysis

Analysis was performed with Sigmaplot 14 (Systat software, San Jose, CA) and Prism 7.0 (Graphpad, La Jolla, CA). Animal numbers were estimated on the basis of power analysis, past experience with each type of study, and published reports. Statistical data were presented as mean ± S.E.M. All comparisons among animal groups were tested for significance using one-way ANOVA with repeated measures followed by Bonferroni's *post-hoc* tests, or repeated measures two-way ANOVA followed by Sidak's multiple comparison tests. Abnormal mitochondria incidence among different group of animals was compared using Chi-square tests. For all statistical analyses, the α level is 0.05, $P < 0.05$ was considered to be statistically significant. Statistically significant differences were indicated in each figure (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). The n in all experiments is the number of rats tested in each condition except where otherwise indicated.

3. Results

3.1. The expression level of KCNQ channels in primary sensory neurons

KCNQ channels are widely expressed in the primary sensory neurons [49, 55]. However, their expression in primary sensory neurons was suppressed in some chronic pain models [55, 72]. As an ideal target for PIPN treatment, one of the requirements is that there are enough channels remaining during PIPN development. Thus, we tested whether the expression of KCNQ channels are altered by treatment with paclitaxel. As KCNQ2, KCNQ3, and KCNQ5 are the major subtypes expressed in DRG neurons [49, 55], we found that the protein level of KCNQ5 in DRGs was unchanged after paclitaxel plus brief vehicle or retigabine treatment, as revealed by Western blot analysis. Although we were able to measure KCNQ5 protein levels in DRGs by Western blot, we did not find reliable antibodies against KCNQ2 and KCNQ3 to measure their protein levels. Instead, the qRT-PCR analysis was used to measure mRNA expression of KCNQ2 and KCNQ3 in DRGs. We found that paclitaxel treatment did not significantly alter KCNQ2, KCNQ3, and KCNQ5 mRNA levels in the L4 and L5 DRGs as compared to the levels from sham treated animals (Fig. 1). We then tested the expression of KCNQ channels after repeated retigabine application with paclitaxel. Retigabine did not affect significantly mRNA expression of either KCNQ2, KCNQ3, or KCNQ5 compared to control groups and paclitaxel-treated group respectively. Thus, these data indicate that the

expression of KCNQ channels are not altered following paclitaxel treatment with or without brief retigabine co-administration.

3.2. Combining retigabine with paclitaxel mitigates the development of neuropathic pain.

Recent evidence suggests that paclitaxel can acutely excite human primary sensory neurons [39]. In addition, patients with paclitaxel-induced acute pain are usually at high risk of developing PIPN [42, 53]. These results suggest that an acute increase in the excitability of nociceptors could underlie the chronic alterations of PIPN. We therefore co-delivered retigabine with paclitaxel to animals and measured behaviors reflective of nociceptor function. Behavioral pretests of intact animals were performed prior to paclitaxel treatment. Animals were then counterbalanced to different groups to ensure an equal starting point for the experiment. From day 1 to 10, animals were treated with paclitaxel plus brief retigabine (10 mg/kg in 1 ml saline, i.p.) or its vehicle twice daily. The animals received posttests 12, 28, and 35 days after initial treatment with paclitaxel (Fig. 2A). While paclitaxel induced mechanical hypersensitivity when brief vehicle (for retigabine) was co-administrated, mechanical hypersensitivity was significantly alleviated in animals when brief retigabine was co-administrated with paclitaxel at all time points tested (Fig. 2B). As compared to the paclitaxel plus brief vehicle treatment group, the hindpaw withdrawal threshold to *von Frey* stimulation was significantly increased in the brief retigabine plus paclitaxel group at three different time points (Fig. 2B). Cancer patients who developed PIPN usually report spontaneous pain, so we determined whether the development of spontaneous pain in PIPN animals was also attenuated by early brief application of retigabine using the conditioned place preference test (CPP) 28 days after initial paclitaxel treatment [61, 66]. Similar to what we reported previously [61], no significant effects on CPP were found in sham animals when 3 daily conditioning retigabine (as analgesic) injections were paired with placement in a white chamber and identical conditioning saline injections were paired with the black chamber. However, the paclitaxel-treated rats that received early brief applications of the vehicle (paclitaxel + vehicle group), but not brief retigabine (paclitaxel + retigabine group), developed a significant preference for the innately less-preferred white chamber after pairing conditioned analgesic injections with placement in this chamber (Fig. 2C). These data suggest that brief application of retigabine during paclitaxel exposure attenuates the development of spontaneous pain and mechanical hypersensitivity.

3.3. Combining retigabine with paclitaxel attenuates the morphological alterations of peripheral neuropathy

Because brief retigabine treatment significantly relieved PIPN associated pain, we were curious whether this was associated with reduced morphological changes characteristic of neuropathy. Thus, after behavioral testing, rats were euthanized and spinal segments L4-L5 along with glabrous skin from hindpaws were excised and processed to assess the effects of brief retigabine treatment on tissue properties. Because spinal cord astrocytes are activated after paclitaxel treatment [8, 69, 70], we used the marker GFAP to see if pretreatment with retigabine decreases the activation of astrocytes in the spinal cord [69]. GFAP expression in the spinal cord from different groups was detected by Western blot. As compared to the sham group, we found that the protein level of GFAP was significantly increased in the spinal cords from the paclitaxel plus brief vehicle group, but not that from the paclitaxel plus

brief retigabine group (Fig. 3A, B). Similar as reported [69, 70], however, we did not detect a significant difference in Iba-1 expression level among sham, vehicle- and retigabine-groups (data not shown). Because peripheral nerves in the epidermal region are particularly likely to degenerate as the result of excessive excitation [7, 62], reducing general excitation may preserve many of these peripheral terminals. Fluorescence staining of glabrous skin sections with PGP9.5, which marks intraepidermal nerve fibers, and collagen IV, which denotes the basal lamina between the dermal/epidermal junction, was performed [7]. Ascending nerve fibers crossing the collagen-stained basal lamina into the epidermis were counted by measuring PGP9.5 density in skin sections. We found that IENF density in skin sections from the paclitaxel plus brief vehicle group were greatly reduced 4 weeks after paclitaxel treatment, whereas brief co-treatment with retigabine significantly attenuated paclitaxel-induced IENF degeneration as compared to the sham group (Fig. 3C and D). These data suggest that repeated application of retigabine during paclitaxel administration attenuates the degeneration of peripheral fibers.

Since mitochondrial abnormalities in primary sensory neurons are linked to paclitaxel-induced peripheral neuropathy [21, 62, 63], we investigated whether retigabine pretreatment mitigates the paclitaxel-induced morphological alterations of mitochondria in primary sensory neurons. In the tibial nerve sections taken from the paclitaxel plus brief vehicle animals, swollen and vacuolated mitochondria were observed in both myelinated and unmyelinated fibers (Fig. 4A and C). However, the incidence of fibers with such abnormal mitochondria was greatly decreased in the nerves dissected from animals treated with paclitaxel plus brief retigabine (Fig. 4B and D). These data suggest that repeated application of retigabine can attenuate paclitaxel-induced mitochondrial abnormalities.

3.4. Retigabine does not alter chemosensitivity of breast cancer cells to paclitaxel

An ideal drug to treat PIPN should attenuates or prevent the pathological alterations with no interference to the chemosensitivity to cancer cells. We thus tested whether retigabine alters the anti-tumor effectiveness of paclitaxel. For these experiments, the viability of the human breast cancer cell line SKBR3 was assessed by mitochondrial enzyme activity using the MTT assay [45]. Administration of 10 μM retigabine showed no obvious effects on cell viability at different times, indicating that retigabine is not toxic to these cells (Fig. 5A). In addition, co-administration of retigabine with 10 nM paclitaxel (the half-maximal inhibitory concentration, IC50) did not affect paclitaxel-induced cell death at 72 hours at any concentration tested (Fig. 5B). Thus, these data suggest that retigabine is unlikely to decrease the chemosensitivity of paclitaxel on breast cancer cells.

4. Discussion

This study has shown that pharmacological activation of KCNQ channels with retigabine in primary sensory neurons could be a useful approach for mitigating the development of paclitaxel-induced peripheral neuropathy (PIPN). Due to the lack of an effective vascular permeability barrier in the DRG [1, 29, 33], sensory neurons in the DRGs have greater exposure to systemically applied paclitaxel compared to central nervous system (CNS) tissue or ventral roots [9, 63]. Several mechanisms for PIPN have been proposed, including

mitochondrial dysfunction [16, 21, 22], microtubule alterations [46, 59, 73], inflammation [40, 50, 51], ion channel modulation [25, 38, 39, 67], and oxidative stress [20]. These mechanisms can directly or indirectly alter the excitability of primary sensory neurons. Patients are usually at high risk of developing PIPN if paclitaxel induces acute pain [42, 53], which suggests that an acute increase in the excitability of nociceptors could underlie the chronic alterations of PIPN. In this study, we show that decreasing neuronal excitability by activating KCNQ channels at an early stage can effectively attenuate both the pathological and behavioral alterations in a PIPN rodent model. It has been reported that activating KCNQ channels can attenuate cisplatin-induced axon conductance impairment and peripheral fiber loss [47]. These studies suggest that neuronal hyperexcitation is a shared mechanism and that neuronal hyperpolarization may mitigate the development of peripheral neuropathy, regardless of the chemotherapy drug used.

KCNQ channels are widely expressed in primary sensory neurons [49, 55] and modulate their excitability [15, 49]. Preventing PIPN by activating KCNQ channels requires that there are a sufficient number of channels available for activation during paclitaxel treatment. This was a significant concern as both bone cancer and peripheral nerve injury, but not spinal cord injury, downregulate KCNQ channel expression in primary sensory neurons [55, 61, 72]. In fact, the expression of several ion channels is altered in the DRG neurons after paclitaxel treatment [25, 38, 39, 67]. KCNQ2, KCNQ3, and KCNQ5 are the major KCNQ subtypes expressed in DRG neurons [49, 55]. Our data indicate that paclitaxel exposure with or without brief retigabine treatment did not decrease mRNA expression of these subtypes or protein expression of KCNQ5 in the primary sensory neurons as compared to that from the sham group, which suggests that a sufficient number of channels remain available during treatment.

Multiple sites along pain pathways are altered in the PIPN rodent model. Most research into mechanisms underlying PIPN has focused on paclitaxel-induced plasticity in the spinal cord and primary sensory neurons, including glia cell activation in the spinal cord, inflammatory activity in the DRG, and degeneration of intraepidermal nerve fibers [8, 40, 50, 51, 62, 68, 69]. Our data demonstrate that decreasing neuronal excitability can reduce both the activation of spinal cord astrocytes and the degeneration of IENF. The link between neuronal over-excitation and neuronal degeneration has been well studied. For example, topical application of capsaicin on human skin results in quick loss of epidermal nerve fibers [48]. Many molecules in sensory fibers required for maintaining their structure and function are thought to be synthesized in, and transported from, DRG somata [13]. Given the long journey from DRG somata to peripheral nerves [13], and the fact that maintaining membrane polarization alone accounts for more than 90% of neuronal energy consumption during their enhanced activity [17, 18], over-excitation of primary sensory neurons may thus result in energy exhaustion and degeneration of peripheral nerves because molecules required for metabolic loading fail to refresh in time [16]. Thus, over-excitation of primary sensory neurons results in mitochondria dysfunction and affects the longest sensory nerves to the extremities in a “stocking and glove” distribution. Abnormal neuronal activity has an important role in spinal cord astrogliosis and microgliosis [64]. Thus, spinal cord glia cells are activated even though the concentration of paclitaxel is extremely low in the CNS [9, 63]. In addition, activated glial cells enhance neuronal excitability [44]. DRG neurons terminate in the spinal cord, which may

thus be affected by activated glial cells in the spinal cord to generate chronic discharge long after accumulated paclitaxel has dissipated [62, 63]. Interrupting such a glial-neuronal loop is thus critical for preventing the development of PIPN, as shown by our morphological evidence and behavioral tests.

In addition to the interplay between neuronal excitability and inflammation, other mechanisms underlying neuropathy induced by paclitaxel are thought to include the alteration of neuronal function via microtubule effects [46, 59, 73], the perturbation of mitochondrial function [16, 59, 63, 71], alterations in channel expression [67], or the production of reactive oxygen species [4]. It is unknown whether these are causative mechanisms for paclitaxel-induced peripheral neuropathy or common downstream alterations resulting from nonspecific damage to sensory neurons. Our data indicate that at least the alterations of mitochondrial morphology were attenuated when retigabine was briefly co-administrated with paclitaxel.

Decreasing neuronal excitability with calcium and magnesium infusion leads to a neuroprotective effect in the preclinical studies [3, 23], but the effect of this treatment on oxaliplatin chemosensitivity is uncertain [23, 24, 30, 36]. Our data indicate that the KCNQ channel activator retigabine does not decrease the chemosensitivity of the breast cancer cell line SKBR3 to paclitaxel (Fig. 5). This suggests that retigabine might be a valuable approach to lessen PIPN in human patients.

In our study, only male rats were studied to remove the effect of estrus cycle stage on the treatments. While gender has no obvious effect on the development of paclitaxel-induced mechanical allodynia, it does affect the analgesic effect of low dose ketamine [31]. The correlation between the risk of developing PIPN and paclitaxel-induced acute pain also occurs in female patients [53]. There is no sex-related difference in the pharmacokinetics of retigabine [26], and retigabine dose-dependently inhibits bladder mechano- and nociceptive transduction of female SD rats [2]. The neuroprotective effect observed in male rats in this study can thus be likely extended to female animals or female patients.

Several drugs failed to prevent/attenuate paclitaxel-induced neuropathy in clinical trials despite their promising preclinical observations [28, 35, 37]. This may be due to the fact that reflexive signs other than spontaneous pain were often used to assess pain in these preclinical studies. However, reflexive signs of hypersensitivity are not necessarily correlated with pain levels in patients with neuropathy [34]. Thus, in addition to reflexive tests, we performed conditioned place preference tests to assess spontaneous pain. Another possible reason underlying the different outcomes in preclinical studies and clinical trials could be that most preclinical studies were performed using healthy animals. Thus, the expression levels of treatment targets could be changed in cancer animals. In this regard, KCNQ2/KCNQ3 expression levels in primary sensory neurons from bone cancer animals are decreased [72]. Thus, in the future we will extend this study to animals bearing breast cancer. Our findings are consistent with evidence that retigabine suppresses cisplatin-induced peripheral neuropathy [47], and encourage the exploration of retigabine to treat chemotherapy-induced peripheral neuropathy. It also provides an impetus for the identification of new classes of KCNQ activators for this purpose.

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HIGHLIGHT

- Early repeated application of retigabine attenuates the development of paclitaxel-induced peripheral neuropathy.
- Enough KCNQ channels are readily available for targeting during treatment.
- Retigabine show no obvious interference to the chemosensitivity of cancer cells to paclitaxel.

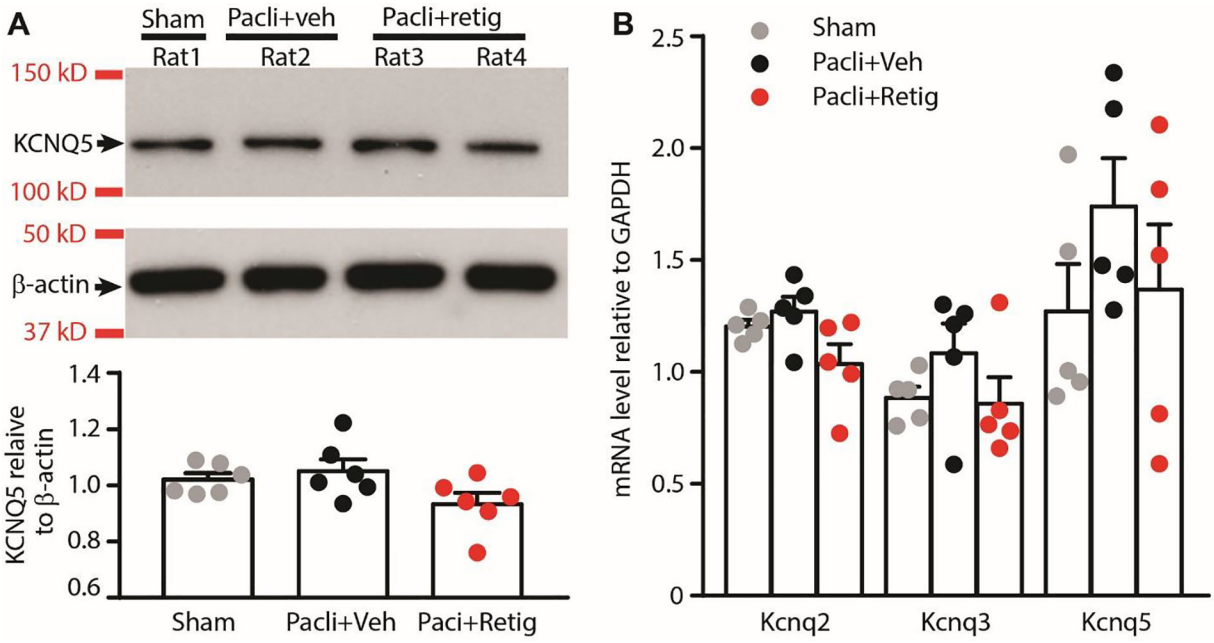

Figure 1.

KCNQ channel expression in DRGs after repeated application of paclitaxel and retigabine. (**A**) Western blot analysis showing lack of effect of paclitaxel and retigabine on KCNQ5 protein expression in L4 and L5 DRGs. Upper panel: Bands of KCNQ5 and β-actin probed with specific antibodies. Lower panel: relative protein expression levels of KCNQ5 compared to β-actin. P = 0.089, $F_{(2,15)}$ = 2.855, one-way ANOVA with repeated measures followed by Bonferroni's *post-hoc* tests. Dots in columns represent one animal each. Six animals were used for each group. (**B**) qRT-PCR analysis showing Kcnq2, Kcnq3 and Kcnq5 mRNA levels in L4 and L5 DRGs 28 days after initial paclitaxel/retigabine treatments. All data are normalized to GAPDH. Dots in columns represent one animal each. P = 0.0669, $F_{(2,12)}$ = 3.418 for KCNQ2; P = 0.2859, $F_{(2,12)}$ = 1.329 for KCNQ3; P = 0.3790, $F_{(2,1)}$ = 1.053 for KCNQ5. One-way ANOVA with repeated measures followed by Bonferroni's *post-hoc* tests. Five animals were used for each group, and the experiments were performed twice independently. Pacli, paclitaxel; retig, retigabine.

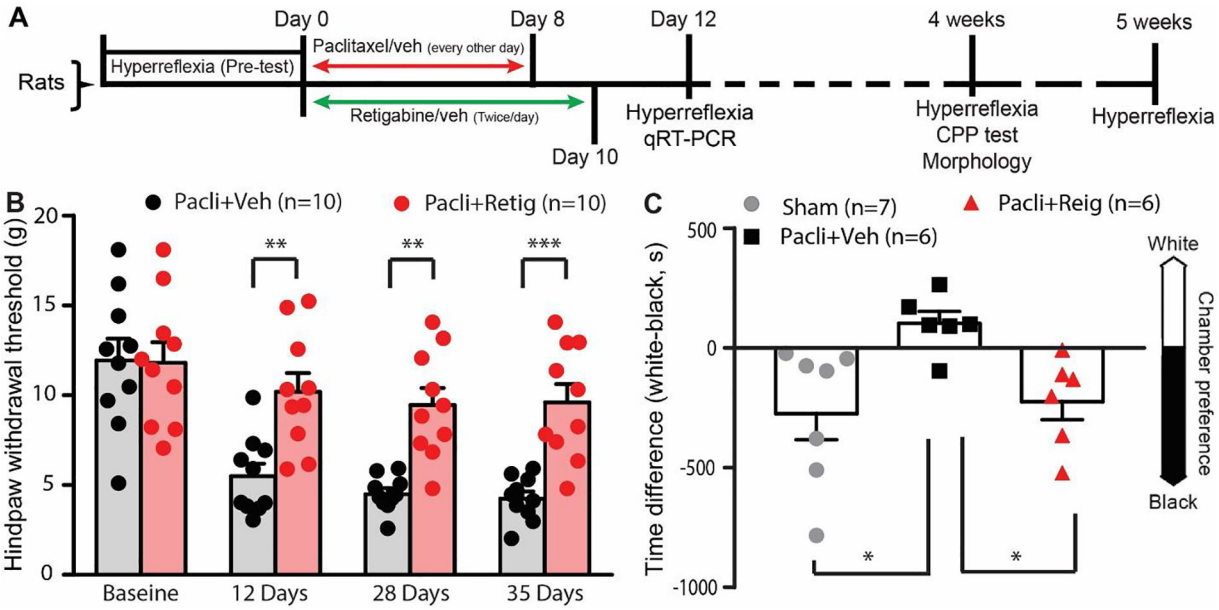

Figure 2.

The effect of early repeated application of retigabine on the development of chronic behavioral hypersensitivity and spontaneous pain after paclitaxel. (**A**) Timeline for the experiments. (**B**) Mechanical hypersensitivity of hindpaws was measured 12, 28, and 35 days after initial paclitaxel treatment. Dots in columns represent one animal each. Repeated measures two-way ANOVA followed by Sidak's multiple comparison test (treatment $F_{(1, 9)} = 21.58$, $p = 0.0012$; time $F_{(3, 27)} = 34.16$, $p < 0.0001$; interaction $F_{(3, 27)} = 4.546$, $P = 0.0105$. Baseline, $P > 0.999$; 12 days, $P = 0.0024$; 28 days, $P = 0.0014$; 35 days, $P = 0.0006$. (**C**) Conditioned place preference tests were performed 4 weeks after treatment. Dots in each column represent individual rats tested in each condition. $P = 0.039$, $F_{(2, 16)} = 4.0$, One-way ANOVA, Pacli, paclitaxel; retig, retigabine. *, $p<0.05$.

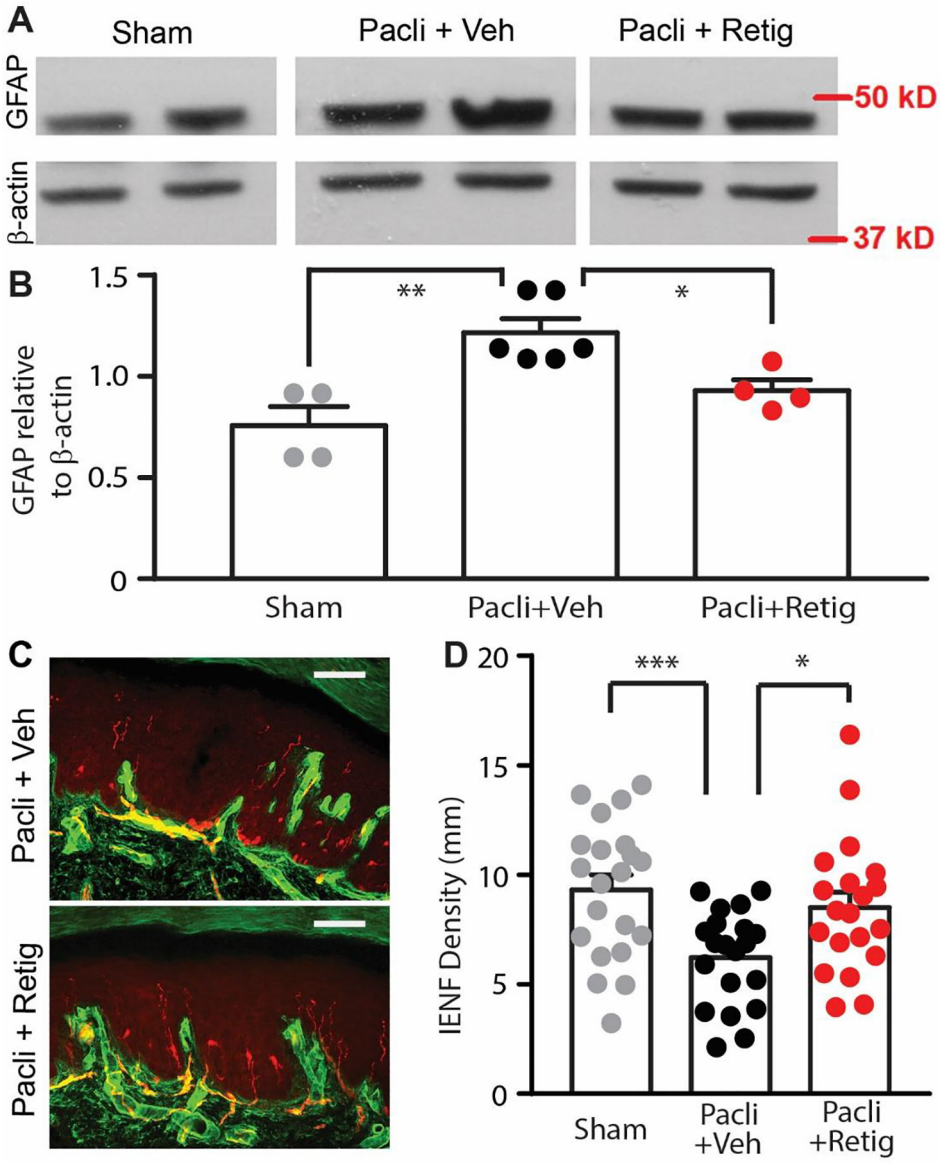

Figure 3.

The effect of early, repeated application of retigabine on the paclitaxel-induced activation of astrocytes in the spinal cord and degeneration of peripheral nerves. (**A**) Representative images showing the expression of GFAP in L4/L5 spinal cords from sham, paclitaxel with vehicle, and paclitaxel with retigabine groups. Shown is a representative experiment from 3 independent experiments. (**B**) Quantification of GFAP expression normalized to β-actin in L4/L5 spinal cords. N shows the number of animals tested. P = 0.02, $F_{(2,13)}$ = 11.11, one-way ANOVA. (**C**) Representative images showing PGP9.5-stained intraepidermal nerve fibers (red) that crossed the collagen-stained dermal/epidermal junction (green) in the skin sections from paclitaxel with vehicle and paclitaxel with retigabine groups. Scale bars, 100 μm. (**D**) The effect of co-treatment with retigabine/vehicle on paclitaxel-induced PGP9.5-positive fiber loss in the epidermis. Dots in each column represents a section. 4–5 sections

per animal were assessed. 4 sham, 5 brief vehicle-treated, and 5 brief retigabine-treated animals were used. P = 0.003, $F_{(2,58)} = 6.567$, one-way ANOVA.

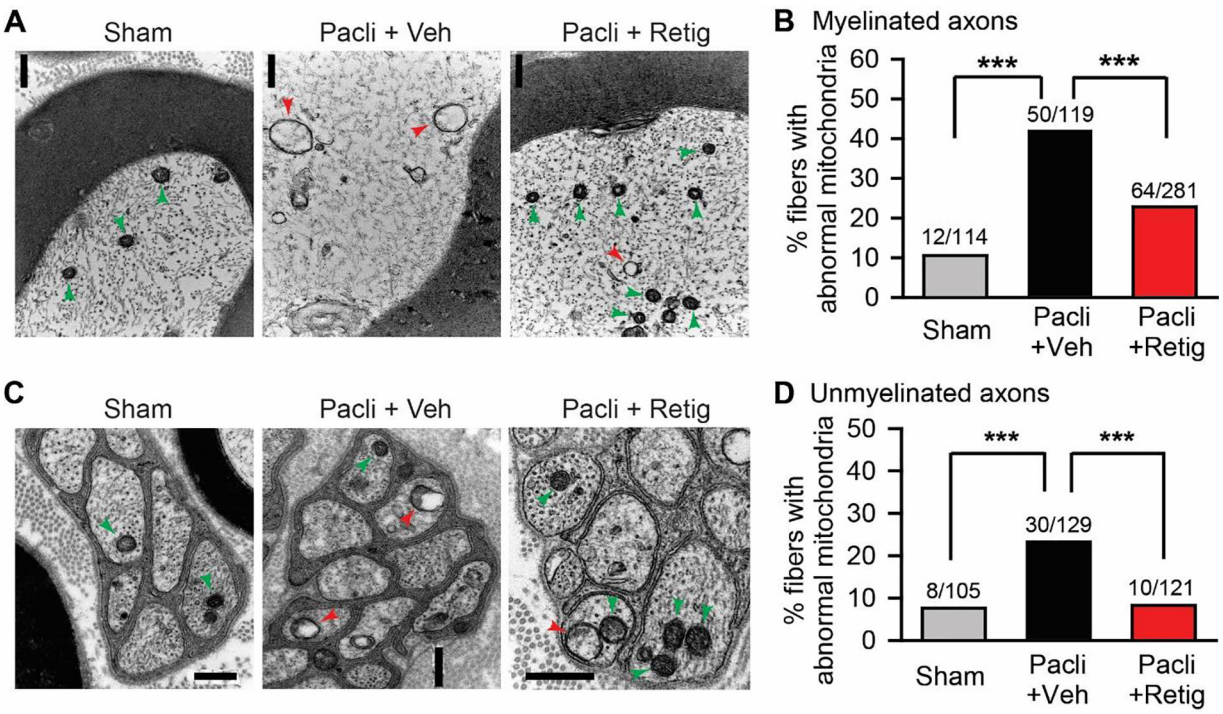

Figure 4.

The effect of early, repeated application of retigabine on the morphological alteration of mitochondria in tibial nerves after paclitaxel. Representative images of swollen mitochondria (red arrows) with vacuoles and small oval mitochondria (green arrows) with intact double membranes in myelinated axons (**A**) and unmyelinated axons (**C**) of tibial nerve sections from sham (left panel), paclitaxel plus vehicle (middle panel), and paclitaxel plus retigabine (right panel) groups. Scale bars, 0.5 μm. (**B**) and (**D**) Quantification of abnormal mitochondria in both myelinated axons and unmyelinated axons. P = 0.0003, X^2 = 16.442 for unmyelinated fibers; P < 0.0001, X^2 = 32.213 for myelinated fibers; Chi-square tests. N is the number of fibers tested. Four animals for each group were used. Data were collected 28 days after initial paclitaxel treatment. *, p<0.05; **, P<0.01, ***, P<0.001.

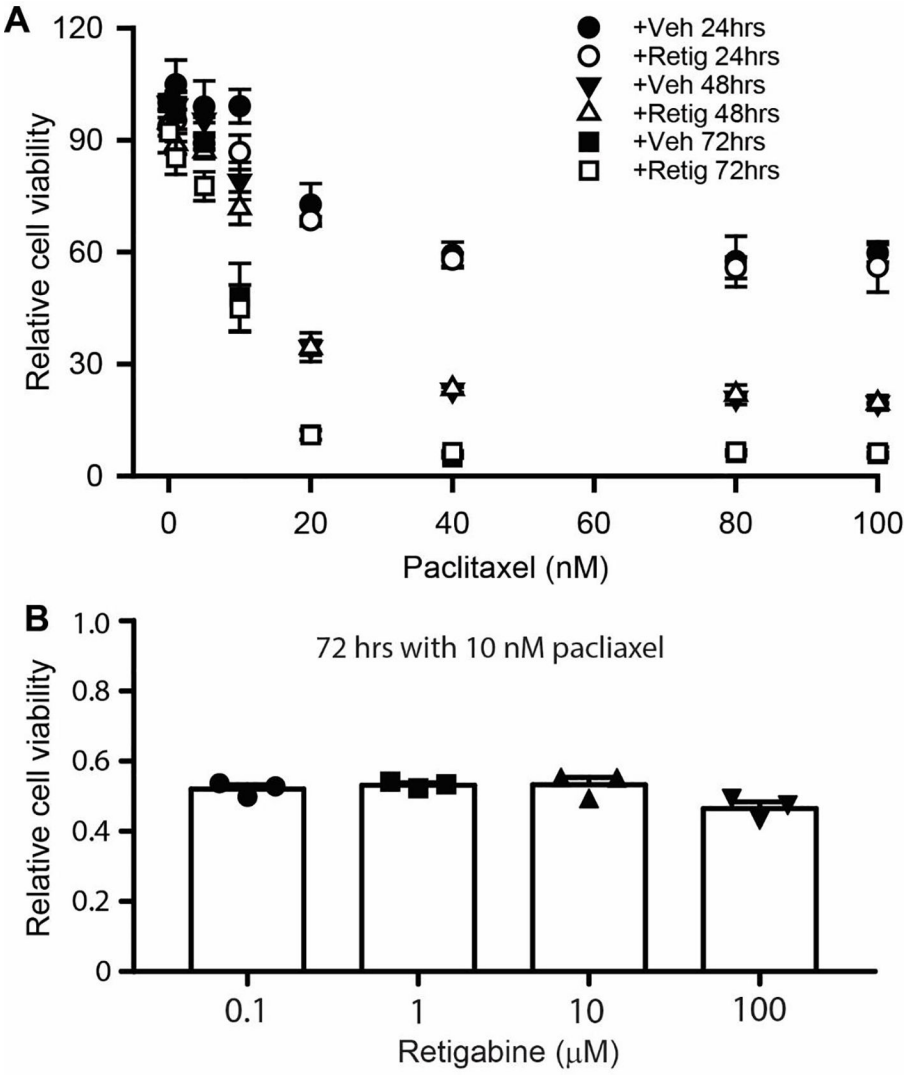

Figure 5.

The effect of retigabine on the chemosensitivity of SKBR3 breast cancer cells to paclitaxel. (**A**) MTT assay of SKBR3 cells treated with 10 μM retigabine plus different concentrations of paclitaxel (0 – 100 nM) at 24 hrs, 48 hrs, and 72 hrs. (**B**) MTT assay of SKBR3 cells treated with 10 nM paclitaxel plus different concentration of retigabine at 72 hrs. P = 0.09, $F_{(3,6)} = 3.487$, one-way ANOVA. Dots in each column represent one independent experiment. Each experiment was performed three times.