# Corydalis Saxicola Bunting Total Alkaloids Inhibits Paclitaxel-Induced Peripheral Neuropathy By Regulating PKCε-TRPV1 and p38 MAPK-TRPV1 Signaling Pathways

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**Research**

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# Abstract

**Background:** *Corydalis saxicola* Bunting, a traditional Chinese medicine, has been proven to work well in anti-inflammation, blood circulation improvement, hemostasis, analgesia. This study was designed to observe the effects and potential mechanism of *Corydalis saxicola* Bunting total alkaloids (CSBTA) on paclitaxel-induced peripheral neuropathy (PIPN).

**Materials and methods:** Following 4 times intraperitoneal injections of paclitaxel (2 mg/kg) and intragastrically (i.g.) administrated at 30 or 120 mg/kg CSBTA, mechanical and thermal allodynia and hyperalgesia in rats were tested. After 40 days, serum was collected for the detection of PGE2, TNF-α, and IL-1β by ELISA. The L4-L6 segment spinal cord, DRG, and plantar skin were harvested, and protein and gene expression of CGRP, SP, TRPV1, p38, and PKCε were analyzed by Western-blot or RT-qPCR. Parallelly, the PIPN cell model was also established in primary DRG neurons by paclitaxel stimulation (300 nM, 5 d). We examined PGE2, TNF-α and CGRP mRNA levels, and the protein expression on the PKCε-TRPV1 and p38 MAPK-TRPV1 pathways in PIPN cell model with or without CSBTA (25 μg/ml and 50 μg/ml).

**Results:** The results showed that CSBTA effectively ameliorated allodynia and hyperalgesia in PIPN rats, regulated the contents of cytokines and neuropeptides in different tissues and cell models. CSBTA significantly decreased the protein expression of PKCε-TRPV1 and p38 MAPK-TRPV1 signaling pathways in the spinal cord and DRG tissues in the PIPN animal model and primary DRG neurons.

**Conclusion:** Therefore, CSBTA has a perspective therapeutic effect on the treatment of paclitaxel-induced peripheral neuropathy.


# Introduction

Paclitaxel, extracted from Pacific yew tree *Taxus brevifolia* Nutt., is widely used in the treatment of solid tumors, such as breast cancer, lung cancer, ovarian cancer [1]. It exerts anticancer effects by increasing the stability of tubulin polymer and inhibiting the proliferation of cancer cells. However, paclitaxel-induced peripheral neuropathy (PIPN), a serious side effect of paclitaxel, severely limits paclitaxel's clinical application and is characterized by numbness, tingling, spontaneous pain, and temperature sensitivity. The incidence of PIPN increases significantly during continuous treatment, and PIPN persists for months or years after the end of paclitaxel treatment [2]. PIPN responds poorly to analgesics such as opioid analgesics, anticonvulsants, or antidepressants [3]. Therefore, there is no clear preventive treatment, and the underlying mechanism is still unclear.

Major mechanisms of PIPN include mitochondrial damage, disruption of axonal transport, neuronal inflammation, and increased ion channel activity on the peripheral sensory neurons [4-5]. Currently, some studies have emphasized the role of transient receptor potential vanilloid type 1 (TRPV1) in the development of PIPN [6-8]. TRPV1, one of the nonselective cation channels, could be activated by heat (43 °C), proton, and ligand (e.g., capsaicin) [6], and is one of the major mechanisms associated with inflammatory and neuropathic pain [9-11]. TRPV1 is transported from DRG along the sciatic nerve to

peripheral nerves of the skin, and it is also transferred from DRG to the dorsal horn of the spinal cord [12]. Hara et al have confirmed that intraperitoneal injection of paclitaxel could increase the TRPV1 level in rat plantar skin and sensitize the TRPV1 channel which is believed to be the cause of heat hyperalgesia [7]. TRP channels are often considered to be downstream of PKC signaling and linked with paclitaxel-induced pain behavior. There are three isoforms of PKC that are co-labeled with TRPV1 on nociceptors [6,13]. Among these isoforms, PKCε is the most dominant in paclitaxel-induced neuropathy [14]. Mitogen-activated protein kinases (MAPK) convert extracellular stimuli into a variety of intracellular reactions by changing the modification of target proteins after transcription and translation. p38, a kind of MAPK, is activated by cellular stress and cytokines. Activation of p38 in DRG can increase translation and transport of TRPV1 to the terminal of the peripheral nociceptor, contributing to the maintenance of thermal hyperalgesia [12].

*Corydalis saxicola* Bunting, affiliated to the Papaveraceae Juss., is the whole grass of the perennial herb. Given the function of clearing away heat, detoxifying and dampness, relieving pain, and stopping bleeding, *Corydalis saxicola* Bunting has been widely applied in treating hepatic diseases such as hepatitis, liver cirrhosis, and liver cancer in the clinic [15]. *Corydalis saxicola* Bunting consists of a variety of protoberberine type I alkaloids, including dehydrocavidine, berberine, tetrahydropalmatine, (±)cavidine, (±)tetrahydropalmatine, protopine, etc, which are collectively called *Corydalis saxicola* Bunting Total Alkaloids (CSBTA) [16,44]. Subcutaneous injection of 50 mg/kg CSBTA tended to restore the writhing response in mice and had a significant inhibitory effect when the dose reaches 100 mg/kg. 100 mg/kg CSBTA also increased the threshold for thermal stimulation in a tail-flick test [16]. Our group has previously found that oral administration of CSBTA (30 mg/kg, 60mg/kg, and 120 mg/kg) obviously relieved cisplatin-induced neuropathic pain, and its underlying analgesic mechanism may be to decrease the phosphorylation p38 and its downstream TRPV1 [17]. However, there is no report on the therapeutic action or molecular targets of CSBTA in PIPN.

This study aimed to investigate the effect of CSBTA on alleviating pain behavior and inflammatory status in the rat model of PIPN. Based on current reports, it is assumed that the PKCε-TRPV1 and p38 MAPK-TRPV1 signaling pathways participate in the therapeutic process. Therefore, the purpose of this study was to observe the therapeutic effect of CSBTA on PIPN and explore its mechanism by animal and cell experiments using different pharmacological tools.

# Materials And Methods

### Reagents

The commercial paclitaxel injections (Paclitaxel Injection®, Lot: H99404910) were purchased from SichuanTai Ji Group Co., Ltd (Chengdu, China). CSBTA (Yanhuanglian total alkaloids capsules, Lot: 160701) was kindly supplied by Nanjing Zhongshan Pharm Co., Ltd (Nanjing, China). Sodium carboxymethyl cellulose (CMC-Na) was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). CSBTA powder was suspended in CMC-Na solution in advance. The quality

specification of the CSBTA capsule is controlled by the content of total alkaloids (calculated by dehydrocavidine) and the amount of dehydrocavidine, palmatine, and berberine. Referring to the standard sample of dehydrocavidine, palmatine, and berberine (supplied by National Institutes for Food and Drug Control, Beijing China), the total alkaloids fraction was analyzed as 58% by HPLC-UV analysis.

## Animals

Adult male Sprague-Dawley rats (180 ± 20g) were supplied by the Laboratory Animal Center of Nanjing Qinglongshan (Agreement Number: SCXK-zhe-2014-0001). All rats were maintained under the standard conditions in China Pharmaceutical University Laboratory Animal Center under the12 h dark-light cycles' in the humanized environment (temperature, 23 ± 2 °C, and humidity, 50 ± 10%) with normal chow diet and tap water *ad libitum*. After acclimation for a week, the rats were used for the following experiments.

## Grouping and administration

Rats were randomly divided into 4 groups: blank group (physiological saline & 0.5% CMC-Na solution), paclitaxel group (2 mg/kg paclitaxel & 0.5% CMC-Na solution), 30mg/kg CSBTA group (2 mg/kg paclitaxel & 30 mg/kg CSBTA suspended in CMC-Na solution) and 120 mg/kg CSBTA group (2 mg/kg paclitaxel & 120 mg/kg CSBTA suspended in CMC-Na solution). Except for the blank group, rats were intraperitoneally injected with 2 mg/kg paclitaxel (Paclitaxel Injection®, Tai Ji, Chengdu, China) at 1, 3, 5, and 7 days, and the blank group was intraperitoneally given the corresponding volume of physiological saline [18]. From the first day of the experiment, oral administration of CSBTA with different dose concentrations (30, 120 mg/kg) were given[17], while the blank group and the model group were administered with a corresponding volume of 0.5% CMC-Na solution. During the experimental period, the body weights were recorded daily before drug administration to evaluate the potential effect of CSBTA on body weight changes.

Ahead of the final experiments, the food was deprived overnight (free access to water). After mice were anesthetized, blood was collected from the abdominal aorta, centrifuged at 3000 rpm for 10 min to be harvested plasma for the determination of PGE2, TNF-α, and IL-1β. L4-L6 segment spinal cord, DRG, and plantar skin were harvested and immediately stored at -80 ℃ till analysis**.**

## Mechanical hyperalgesia—Von-Frey test

The mechanical pain threshold of rats was detected by Von-Frey filament according to the "up and down" method initiated by Chaplan[19]. As described previously[17], the rats were individually placed in a transparent plexiglass box with a cover at the top and a reticular structure at the bottom. Every rat was subjected to an adaptive test one week before the formal experiment. After adaptation for 15 min, the mechanical pain threshold was measured when rats were in a quiet state and the movement of walking and scratching disappeared. Von-Frey filament (Stoelting Company, Chicago, USA, stimulus intensity range: 4-180 g) was used to prick the middle area of the ipsilateral posterior toe through the bottom mesh. Initiating from the moderate intensity, the fiber needle vertically pressed the plantar skin until the

needle was warped into a "C" shape. If retraction, movement, or lameness of the hind paw appeared, a positive reaction "X" was designated and the corresponding value was recorded. And then, the strength nearest to low-intensity t was used to stimulate, and negative reaction "O" was recorded if there were no response after stimulus for more than 5 s. Sequentially, the closest high-intensity was continued to stimulate till the rats showed positive behavior. Stimulation was conducted every 2 min and repeated six times. Mechanical hyperalgesia was tested by a double-blind test. Followingly, the "X" and "O" sequences could be obtained and the mechanical pain threshold could be finally calculated. The mechanical pain threshold test in each rat was repeated three times to calculate the average response.

### Thermal sensitivity—Laser thermal pain meter

PL-200 laser thermal pain meter (PL-200, Taimeng Technology, Chengdu, China) was used to detect heat hyperalgesia to assay latent period of heat-stimulated retraction reflex. As described previously [17], each animal was subjected to an adaptive test one week before the formal experiment. Under constant ambient temperature, rats were placed in an observation box for 15 min in advance to make them accommodative and tranquil. Afterward, the optical source (intensity at 35%) was moved to plantar, and the time (from the initial irradiation) was recorded when thermal pain reaction (contracting claw, licking foot, and scratching) occurred. The cutoff time was 25 s to prevent possible injury from high temperature. The thermal sensitivity test was repeated three times at least 5 min intervals in each rat, and the average was applied for data analysis. Thermal sensitivity was tested by a double-blind test.

### Thermal sensitivity—Tail immersion assay

Half an hour after drug administration, rats were subjected to a tail-flick test. As described previously [18], each animal was subjected to an adaptive test one week before the formal experiment. After the rat was no longer struggling, the tail was immersed into a water bath, and time from immersion to tail flick reaction was recorded. The temperature was set at 47 ℃, which was adopted by the tail-flick time lasting 10s for most rats. To prevent the injury from high temperature, the cutoff time was 20 s. Each rat was tested three times with at least 5 min interval, and the average of the three tests was used for data analysis. Thermal sensitivity was tested by a double-blind test.

### Thermal sensitivity—Cold hyperalgesia

Similarly, rats were subjected to a cold hyperalgesia test 30 min after drug administration. As described previously [20,21], dry ice was used to assay cold pain threshold of rats, and the results were expressed as the first occurrence time of paw withdrawal, lameness, scratching, or other reactions in rats after cold stimulation. The room's temperature was kept stable, and rats were placed in an observation box for 15 min to make them quiet. After the dry ice placing on plantar, time from the beginning to the emergence of contracting claw, licking foot, or scratching was recorded. The cutoff time was 90 s to avoid skin cold injury. At least 5 min interval was performed. Each animal was subjected to an adaptive test one week before the formal experiment. Thermal sensitivity was tested by a double-blind test.

### TNF-α, IL-1β, and PGE2 in serum and plantar skin tissues

After 12 h of the last dosing, rats in each group were fasted for 10 h (free access to water) and then anesthetized. Blood was rapidly withdrawn from the abdominal aorta to clean EP tubes. Serum was harvested after centrifugation at 3000 rpm for 10 min. The harvested plantar skin tissues from corresponding rats were immediately shaved on the ice. A piece of 1 g tissue was collected and cut into pieces. Subsequently, they were soaked in 5 ml physiological saline after a gentle vortex at 4 ℃ for 2 h. Afterward, the mixtures were centrifuged at 3000 rpm for 10 min at 4 ℃, and the harvested supernatant was stored in -80 ℃ till analysis [17]. The levels of TNF-α, IL-1β, and PGE2 in obtained plantar skin supernatant and serum were separately detected by ELISA (Calvin Biotechnology, Suzhou, China) according to kit instructions.

### Primary DRG neurons cell culture

As described previously [22,23], the back skin of rats was quickly cut with surgical scissors to isolate the spine after anesthesia. DRG tissue was extracted and placed in a tube containing MEM medium (Gibco, USA) without fetal bovine serum (FBS, Gibco, USA). Subsequently, an aliquot of 2 ml collagenase A (Roche, USA, 1 mg/ml) was added to digest cells for about 90 min. Then, the digestion solution was replaced by 0.25% trypsin (Gibco, USA) for another 20 min. Finally, the digestion was terminated by the MEM medium containing 10% FBS. MEM medium was mechanically triturated with a 1 ml pipette until the solution turned milky. After filtering through a 70 μm cell strainer, the solution was centrifuged at 1000 rpm for 5 min. After removing the supernatant, the remaining cell pellets were resuspended in MEM medium containing 10% FBS and 1% penicillin and streptomycin mixture (Gibco). Eventually, cell suspensions were divided into a six-well plate (~60,000 per well) covering with poly-D-lysine at the bottom (100 μg/mL, Sigma, USA). After culturing for 24h, MEM medium was replaced with neuronal growth medium (Neurobasal medium: B27 = 50:1, both purchased from Gibco, USA), and 1% other agents including penicillin and streptomycin mixture (Gibco), L-Glutamine (0.1 mg/mL, Sigma-Aldrich), and cytarabine (5 μg/mL, Sigma Aldrich) was added. Medium with supplements was changed every two days.

### Cell viability assay

The cell suspension was injected into a 96-well plate and cultured in a 5% CO2, 37 ℃ incubator. After three days, CSBTA group was treated with 0, 0.05, 0.5, 5, 50 μg/ml of CSBTA, and paclitaxel groups were given 0, 1, 10, 100, 500, 1000nM paclitaxel (A4393, APExBIO, USA). Five days later, Cell Counting Kit-8 (CCK-8) solution (Dojindo, Japan) was added. After incubation for 4 hours, absorbance at 450 nm was measured with a microplate reader.

### Drug treatment

After cultured three days, primary DRG neuron cells were equally divided into following five groups: blank group (phosphate buffer saline), paclitaxel group (300 nM paclitaxel), 25 μg/ml CSBTA group (300 nM

paclitaxel + 25 μg/ml CSBTA), 50 μg/ml CSBTA group (300 nM + 50 μg/ml CSBTA) and PKCε inhibitor group (300 nM paclitaxel + 100 nM Staurosporine(A8192, APExBIO, USA)). The co-incubation lasted 5 days and cells were processed as the preparation procedure.

### Western blot analysis

As described previously [17], the spinal cord and DRG were lysed in lysis buffer (RIPA: PMSF: phosphatase inhibitor = 100:1:1) at 1:5 (mg·μL-1) and vigorously crushed by glass homogenizer on ice. An appropriate aliquot of lysis buffer was added and cells were scrapped by cell scraper on ice. The homogenates were immediately centrifuged at 12000 rpm for 20 min at 4 ℃. Protein quantification was performed according to the procedure of the Bicinchoninic Acid Protein Assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Subsequently, extracted protein samples (6×loading buffer: supernatant=1:2, v/v) were boiled for 15 min and stored at -80℃ till analysis.

Loading protein (50 μg) was electrophoresed and separated on a 10% SDS-polyacrylamide gel (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (0.22 μm, Pall, USA). PVDF membrane was blocked in 5% skimmed milk in TBST buffer (Tris-HCl, 5 mM, pH 7.6, NaCl 136 mM, 0.05% Tween-20) at room temperature for 2 h, followed by incubation with different primary antibodies—p38 (1:1000, Abcam, USA), p-p38 (1:1000, Cell Signaling Technology, USA), PKCε (1:1000, Abcam, USA) and TRPV1 (1:200, Alomone, Israel) in TBST at 4 ℃ overnight. After washed with TBST three times, membranes were further incubated with horseradish peroxides (HRP)-conjugated secondary antibody (1:5000 dilution in TBST) for 2 h at room temperature. Antibody binding was detected by enhanced chemiluminescence (ECL). Imagines were acquired by Bio-Rad and quantified by densitometry analysis using Image J software.

### Real-time qPCR analysis

Referring to the manufacture's instruction, spinal cord and DRG tissue weighing 100 mg were homogenized in 1 ml Trizol reagent (Invitrogen, USA) on ice to extract total RNA. While cells were lysed with 1ml Trizol reagent per well to extract total RNA [24]. RT-PCR was proceeded according to HieffTM First Strand cDNA Synthesis SuperMix Kit instruction. β-actin was used as an endogenous control. Quantitative PCR was performed in 20 μl reactions using HieffTM qPCR SYBR Green Mester Mix (No Rox) kit. Primer sequences are listed in Table 1. Relative quantities of the candidate genes and β-actin mRNA were detected by the Bio-Rad image system (Bio-Rad, USA) and calculated by the comparative threshold cycle (Ct) method.

### Statistical analysis

Data were expressed as a mean ± SD (standard deviation) of at least three independent experiments. Statistical analysis was performed using GraphPad Prism software, version 6.0 (San Diego, CA, USA). The statistical significance was analyzed using unpaired Student's t-test, or one-way ANOVA followed by Dunnett's multiple comparison test. A value of $p < 0.05$ was considered to be statistically significant.

# Results

### CSBTA normalized the neurological behavior in PIPN rats

In this experiment, intraperitoneal injection of 2 mg/kg paclitaxel four times caused obvious mechanical, cold and thermal allodynia and hyperalgesia in rats (**Figure 1a**), implying peripheral neuropathy occurrence in SD rats. During the whole experiment period, there was no death occurred. The bodyweight of rats in each group showed a normal increasing trend, but it did not exhibit a significant difference among different groups (**Figure 1b**, $p > 0.05$).

Compared with the model group (paclitaxel), the 30 mg/kg CSBTA group and 120 mg/kg CSBTA group gradually recovered mechanical threshold from the 20th and the 16th day after continuous administration of CSBTA (**Figure 1c**, $^{**}p < 0.01$). There was no significant difference between the 120 mg/kg group and the blank group from the 24th day by Von Frey hair test (**Figure 1c**, $p > 0.05$). Cold pain threshold was gradually improved from the 20th and the 18th day in 30 or 120 mg/kg CSBTA groups (**Figure 1d**, $^{**}p < 0.01$), and there was no significant difference between the 120 mg/kg group and blank group after 20 days (**Figure 1d**, $p > 0.05$).

The response to thermal sensitivity was measured by a laser thermal pain meter and tail immersion assay. Compared with the rats' thermal sensitivity in the model group (**Figure 1e**), rats' thermal threshold was recovered from the 16th day when rats treated with 30 or 120 mg/g CSBTA ($^{**}p < 0.01$) in the laser thermal pain experiment, while two dosing groups exhibited no distinct difference compared with the blank group ($p > 0.05$) from the 24th and the 22nd day. In the tail immersion experiment (**Figure 1f**), thermal sensitivity was gradually restored from the 22nd and the 18th day ($^{**}p < 0.01$) after treating with 30 mg/kg and 120 mg/kg CSBTA group. Compared with the blank group, the 120 mg/kg CSBTA group already had no significant difference from the 24th day ($p > 0.05$). At the last time to measure the behavioral indexes, 120 mg/kg CSBTA group and 30 mg/kg CSBTA group had a significant difference in mechanical pain thresholds ($P<0.01$), but there were no significant differences in thermal sensitivity thresholds ($P>0.05$). All these results indicated that CSBTA made neurological behaviors of PIPN gradually recover to normal.

### CSBTA decreased the levels of IL-1β, TNF-α, PGE2, and neuropeptides in PIPN rats

The inflammatory process activated by chemotherapeutic drugs is considered to be a potential trigger for CIPN, and the release of proinflammatory cytokines or chemokines is regarded as one of the major mechanisms of neuroimmune response [2,25]. The levels of inflammatory factors IL-1β, TNF-α, and PGE2 in serum and plantar skin supernatant were measured using an ELISA kit. As shown in **Figures 2a and 2b**, cytokines in rats treated with paclitaxel were significantly increased compared with those in physiological saline ($^{\#\#}p < 0.01$). However, CSBTA (30 mg/kg) reduced abovementioned inflammatory factors to some extent ($^{*}p < 0.05$), while CSBTA (120 mg/kg) significantly presented more improvements ($^{**}p < 0.01$), especially in the content of serum IL-1β and plantar skin supernatant TNF-α.

SP and CGRP are excitatory neuropeptides released by the primary sensory afferent nerve in the dorsal horn, causing allodynia and hyperalgesia [26]. Several studies have reported that SP and CGRP released from the spinal cord and DRG were involved in PIPN [27]. The levels of inflammatory factors and relevant neuropeptides (SP is synthesized by tachykinin, TAC-1) in the spinal cord and DRG tissues were detected by RT-qPCR, as shown in **Figures 2c and 2d**. The results indicated that the paclitaxel group significantly increased the levels of inflammatory factors (PGE2, TNF-α) and neuropeptides (CGRP and SP) in the spinal cord and DRG compared with the blank group (#p < 0.05 or ## p < 0.01), while 120 mg/kg CSBTA group exerted obvious inhibitory effect on the mRNA levels of those factors (*p < 0.05 or **p < 0.01). Compared with 30 mg/kg CSBTA, 120 mg/kg CSBTA has a better inhibitory effect on inflammatory factors, such as CGRP and TNF-α in the spinal cord, and PGE2 in DRG.

### CSBTA inhibited PKCε-TRPV1 and p38 MAPK-TRPV1 pathways in the spinal cord and DRG

It is widely recognized that paclitaxel could not penetrate the blood-brain barrier. Hence, it does not readily accumulate in the central nervous system. However, it largely damages the peripheral nervous system [28], including nerve terminal, peripheral nerves, and DRG during the development of PIPN. Referring to the reference [18,29] proteins from the spinal cord and DRG from each group were extracted and expressions of PKCε, TRPV1, p-p38 MAPK were analyzed by Western-blot. Figure 3 showed that CSBTA could significantly inhibit the above proteins involving PKCε-TRPV1 and p38 MAPK-TRPV1 pathways by different degrees (*p < 0.05 or **p < 0.01). Compared with 30 mg/kg, 120 mg/kg CSBTA had a more significant inhibitory effect on PKCε in the spinal cord (P<0.05). However, the inhibition effect of 30 mg/kg CSBTA on p-p38 MAPK protein was more obvious than 120 mg/kg CSBTA (P<0.05). There is no significant difference in the DRG between 30 mg/kg and 120 mg/kg CSBTA groups (P>0.05). As a stress-activated protein kinase, p38 MAPK is activated by some environmental stress and cytokines to participate in an inflammatory response. In this experiment, CSBTA reduced the levels of pro-inflammatory cytokines (such as IL-1β, TNF-α, PGE2) via regulating PKCε-TRPV1 and p38 MAPK-TRPV1 pathways and alleviated the symptoms of chronic neuropathic pain in PIPN rats.

### CSBTA reduced the PGE2, TNF-α, and CGRP mRNA levels in paclitaxel-stimulated primary DRG neurons

Before further study, it was necessary to crucially evaluate possible damage to cells along with the increasing dose amounts. The serial concentration gradients of CSBTA and/or paclitaxel were incubated with primary DRG neurons for 5 days, cell viability was measured by the CCK-8 method to optimize the drug concentration. As shown in **Figure 4**, paclitaxel (0~1000 nM) and CSBTA (0~50 μg/ml) exhibited no obvious effect on cell viability (p>0.05), which provided a suitable concentration range in vitro experiment.

mRNA expression of cytokines was detected by RT-qPCR. Paclitaxel up-regulated the expression of PGE2, TNF-α and CGRP in DRG neurons (**Figure 5**, ##p < 0.01), and these cytokines could be inhibited by 25 μg/ml CSBTA, 50 μg/ml CSBTA and 100 nM Staurosporine. 50 μg/ml CSBTA significantly repressed in gene expression of PGE2, TNF-α and CGRP compared with those in model group, *p < 0.05 or **p < 0.01.

### CSBTA regulate the PKCε-TRPV1 and p38 MAPK-TRPV1 pathways in paclitaxel-stimulated DRG neurons

It is widely believed that both PKCε-TRPV1 and p38 MAPK-TRPV1 signaling pathways are involved in the occurrence of paclitaxel-induced chemotherapy pain. Therefore, the analgesic mechanism of CSBTA was explored by observing the two pathways. In **Figure 6**, the paclitaxel group significantly raised the expression of PKCε (**a**), TRPV1 (**b**) and p-p38 protein (**c**) and TRPV1 mRNA level (**d**) in primary DRG neurons compared with the blank group (##p < 0.01), whereas CSBTA (50 μg/ml) and Staurosporine (100 nM) could significantly reduce the expressions of relevant proteins and mRNA (**p < 0.01). These results suggested that CSBTA probably achieved an analgesic effect by inhibiting PKCε-TRPV1 and p38 MAPK-TRPV1 signaling pathways.

## Discussion

Paclitaxel-induced peripheral neuropathy in normal rats is regarded as an internationally recognized animal model of neuropathic pain. PIPN model produces persistent pain behaviors, including mechanical hyperalgesia and temperature hyperalgesia (heat and cold), which will contribute to elucidate the mechanisms [6,30]. In the present study, after 4 times intraperitoneal injections of paclitaxel (2 mg/kg), the mechanical and thermal allodynia and hyperalgesia in rats were changed. As shown in **Figure 1**, 30 mg/kg CSBTA and 120 mg/kg CSBTA gradually recovered mechanical pain caused by paclitaxel after two weeks of administration and eventually normalized. Some studies have shown that paclitaxel can induce thermal hyperalgesia in the PIPN rat model, which is consistent with clinical hyperalgesia in patients with peripheral neuropathy [6,31]. We used a laser thermal instrument and tail-flick test to detect the heat sensitivity of rats. The results showed that 30 mg/kg CSBTA and 120 mg/kg CSBTA could make thermal hyperalgesia of the foot and tail of the rats tend to be normal. Cold-induced pain is often used as an indicator to measure neuropathic pain behavior in animal models, and detection of cold-induced pain is an important method to study the underlying pathophysiological mechanism of pain. In rodents, there are a variety of methods for detecting cold-induced pain, such as the usage of acetone, cold plate experiments, and ethyl chloride spray. Each test has its advantages and disadvantages [32]. In this experiment, we used dry ice to stimulate the feet of rats. The experiment found that 30 mg/kg CSBTA and 120 mg/kg CSBTA restored the threshold of cold hyperalgesia in PIPN rats.

TRPV1, one of the nonselective cation channels, could be activated by heat (43 ℃), proton, and ligand (e.g. capsaicin) [6]. Hara et al [7] have confirmed that intraperitoneal injection of paclitaxel could increase TRPV1 level in rat plantar skin and sensitize the TRPV1 channel which is believed to be the cause of heat hyperalgesia. Some studies have shown that intraperitoneal injection of TRPV1 antagonists (such as capsazepine and SB366791) in PIPN animals would attenuate paclitaxel-induced thermal hyperalgesia in a dose-dependent manner, but exhibited no obvious effect on mechanical allodynia [6], further confirming the relationship between TRPV1 and PIPN thermal hyperalgesia. Inflammatory states or injection of some cytokines (such as bradykinin and nerve growth factor) could develop into severe thermal hyperalgesia, and deletion of such sensitization in TRPV1-/- mice would provide genetic support for the idea that TRPV1 is a key component of inflammation-induced hyperalgesia [33]. In combination with the current

animal experiments and relevant clinical studies of pain, we found that there was no particularly appropriate, available, and universally recognized TRPV1 antagonist drug in the clinical treatment of pain. Therefore, we did not set a positive control group in the animal part of this experiment. Activation of TRPV1 led to depolarization of neurons and the release of neuropeptides in peripheral and central nerve endings. And then, neuropeptides in turn couple with their related receptors and enhance the sensitivity of nociceptors [34]. Excitatory neuropeptides, such as calcitonin gene-related peptide (CGRP), are released from primary afferent nociceptors, which serve as a crucial step in the initiation and development of pain. It can be recognized as a potential biomarker for the activation of sensory neurons [13]. The increased release of neuropeptides induced by paclitaxel may result in sensitization of primary sensory neurons, further caused mechanical allodynia and tingling in patients [35]. Therefore, we used CGRP mRNA as an indicator to observe paclitaxel effects on neuronal function.

Based on the LPS-like effect, paclitaxel causes immune cells accumulation in DRG, increases intracellular Ca2+ levels, and promotes phosphorylation of PKC. The increase of PKC expression in DRG was observed in the PIPN mouse model, and inhibition of PKC attenuated paclitaxel-induced chronic neuropathic pain [28]. PKCε, one of the subtypes of PKC, induces intracellular signaling pathways in primary nociceptor receptors and mediates cytokine-induced nociceptor receptors activation. It is responsible for the transition from acute to chronic pain [13]. PKCε is also a kind of second messengers, which mediates paclitaxel-induced hyperalgesia [36]. Recent studies have shown that one of the targets of PKCε in peripheral nociceptive signaling is TRPV1 [13,37-39]. A variety of inflammatory mediators (e.g., ATP, bradykinin, and prostaglandins) could enhance TRPV1 through the PKC-dependent pathway and reduce the temperature threshold of TRPV1[36].

Mitogen-activated protein kinases (MAPK), mediated by p38 rather than ERK, acts on DRG neurons in the pathogenesis of pain [12]. On the one hand, after p38 MAPK activation in DRG, it would enhance the expression of TRPV1, and promote TRPV1 translation and transporting to peripheral sensors, which is helpful to maintain inflammatory heat hyperalgesia [40]. On the other hand, the p38 MAPK pathway directly or indirectly activates various transcription factors and induces the release of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6. Studies have found that p38 MAPK also plays a pro-inflammatory role, further activating TRP by promoting phosphorylation of intracellular PKC and PKA [12]. Systemic administration of p38 inhibitors exerted anti-inflammatory effects by reducing the synthesis of cytokines TNF-α and IL-1β and the production of PGE2 [12]. Both p38 MAPK and ERK1/2 participate in microglia activation and conduction changes of ion channels, further contributing to the excitability of primary nociceptors and spinal cord neurons after peripheral nerve injury. In general, p38 MAPK is a key regulator in DRG neurons and spinal cord neurons, involving in the progression of PIPN.

In PIPN rats, paclitaxel does not penetrate the blood-brain barrier and therefore does not accumulate in the central nervous system, but damages the peripheral nervous system, including nerve endings, peripheral nerves, and DRG. Paclitaxel may cause damage to the sciatic nerve, nerve endings, and DRG during the development of PIPN [29,41]. The results showed that 120 mg/kg CSBTA could effectively inhibit PKCε-TRPV1 and p38 MAPK-TRPV1 signaling pathway protein expression (**Figure 3**). Accordingly,

paclitaxel increased the level of inflammatory cytokines (such as TNF-α, IL-1β, and PGE2 in serum and plantar skin supernatant) in PIPN rats, and CSBTA exhibited a significant inhibitory effect on these inflammatory cytokines. In the spinal cord and DRG tissues, mRNA levels of PGE2, TNF-α, CGRP, and SP showed the same trend as the above situation (**Figure 2**). These results are consistent with the published results of CSBTA improving cisplatin-induced peripheral neuropathy [17]. According to the results of in vivo experiments, CSBTA alleviates the inflammatory state of animals by inhibiting PKCε-TAPV1 and p38 MAPK-TRPV1 signaling pathway and affects related behavioral indicators, to eventually achieve the purpose of treating PIPN.

It is worth noting that although 30 mg/kg CSBTA significantly improved animal behavior index and PKCε-TRPV1 and p38 MAPK-TRPV1 signaling pathway, inhibition degree of inflammatory cytokines in plantar skin tissues and serum was not as significant as that in the 120 mg/kg CSBTA group. Therefore, what needs to be considered is whether there are other proteins involved besides proteins already detected. The reason why the low-dose group did not improve inflammation as much as the high-dose group is worthy of further research.

Sensory neurons of dorsal root ganglion (DRG) receive signals from peripheral nerve endings and integrate sensory signals to the spinal cord. DRG is isolated from the blood-brain barrier and is surrounded by rich capillaries, making it extremely susceptible to drug neurotoxicity and participating in sensory signal transmission [42]. Although paclitaxel cannot penetrate the blood-brain barrier, it can accumulate in dorsal root ganglion neurons [43]. Primary DRG sensory neurons were exposed to paclitaxel and an in vitro induced peripheral neuropathy model was constructed to help screen and demonstrate whether the drug had a neuroprotective effect on chemotherapy-induced sensory neuronal neurotoxicity. The concentration of paclitaxel and CSBTA did not affect cell viability (**Figure 4**). Consistent with the data obtained from in vivo, 50 μg/ml CSBTA significantly inhibited protein content of PKCε, TRPV1, and p-p38 MAPK, and reduced mRNA levels of PGE2, TNF-α, and CGRP (**Figure 5** and **Figure 6**). Interestingly, although the inhibitory effect of PKCε inhibitors on TRPV1 and PKCε protein is stronger than the inhibitory effect of CSBTA, 50 μg/ml CSBTA has a more obvious downregulation of p-p38 MAPK, suggesting that CSBTA has multiple targets.

# Conclusions

Overall, CSBTA effectively improved behavioral indexes of mechanical and thermal allodynia and hyperalgesia in PIPN rats, and regulated contents of cytokines (such as IL-1β, TNF-α, PGE2) and neuropeptides (SP and CGRP) in different tissues and cell models, and ameliorated pain behavior and inflammatory status of PIPN rats. CSBTA decreased relevant protein expression in PKCε-TRPV1 and p38 MAPK-TRPV1 signaling pathways in the spinal cord and DRG of PIPN animal model, and primary DRG neurons (**Figure 7**). Therefore, CSBTA has a perspective therapeutic effect on the treatment of paclitaxel-induced peripheral neuropathy.

# Abbreviations

CGRP, Calcitonin gene-related peptide; CIPN, Chemotherapy-induced peripheral neuropathy; CSBTA, Corydalis saxicola bunting total alkaloids; DRG, Dorsal root ganglion; IL-1β, Interleukin - 1β; PGE2, Prostaglandin E2; PIPN, Paclitaxel-induced peripheral neuropathy; PKCε, Protein kinase C ε; SP, Substance P; TNF-α, Tumor Necrosis Factor-α; TRPV1, Transient receptor potential vanilloid 1.

# Declarations


### Acknowledgements

Not applicable.


### Authors' contributions

Chu Xue: Conceptualization, Visualization, Investigation, Methodology, Writing - original draft, Writing - review & editing. Si-xue Liu: Formal analysis, Visualization. Jie Hu: Conceptualization, Investigation, Methodology. Jin Huang: Methodology, Visualization. Hong-min Liu: Formal analysis, Writing - review & editing. Zhi-xia Qiu: Methodology,Software, Validation, Resources, Supervision. Fang Huang: Conceptualization, Resources, Data curation, Writing - review & editing, Supervision, Project administration, Funding acquisition.


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### Availability of data and materials

Not applicable.

### Ethics approval and consent to participate

The study was conducted according to the ethical guidelines and approved by the Ethics Committee on Laboratory Animal Management of China Pharmaceutical University.

### Consent for publication

We declare that the Publisher has the Author's permission to publish the relevant contribution.

### Competing interests

The authors declare no competing conflict of interests.

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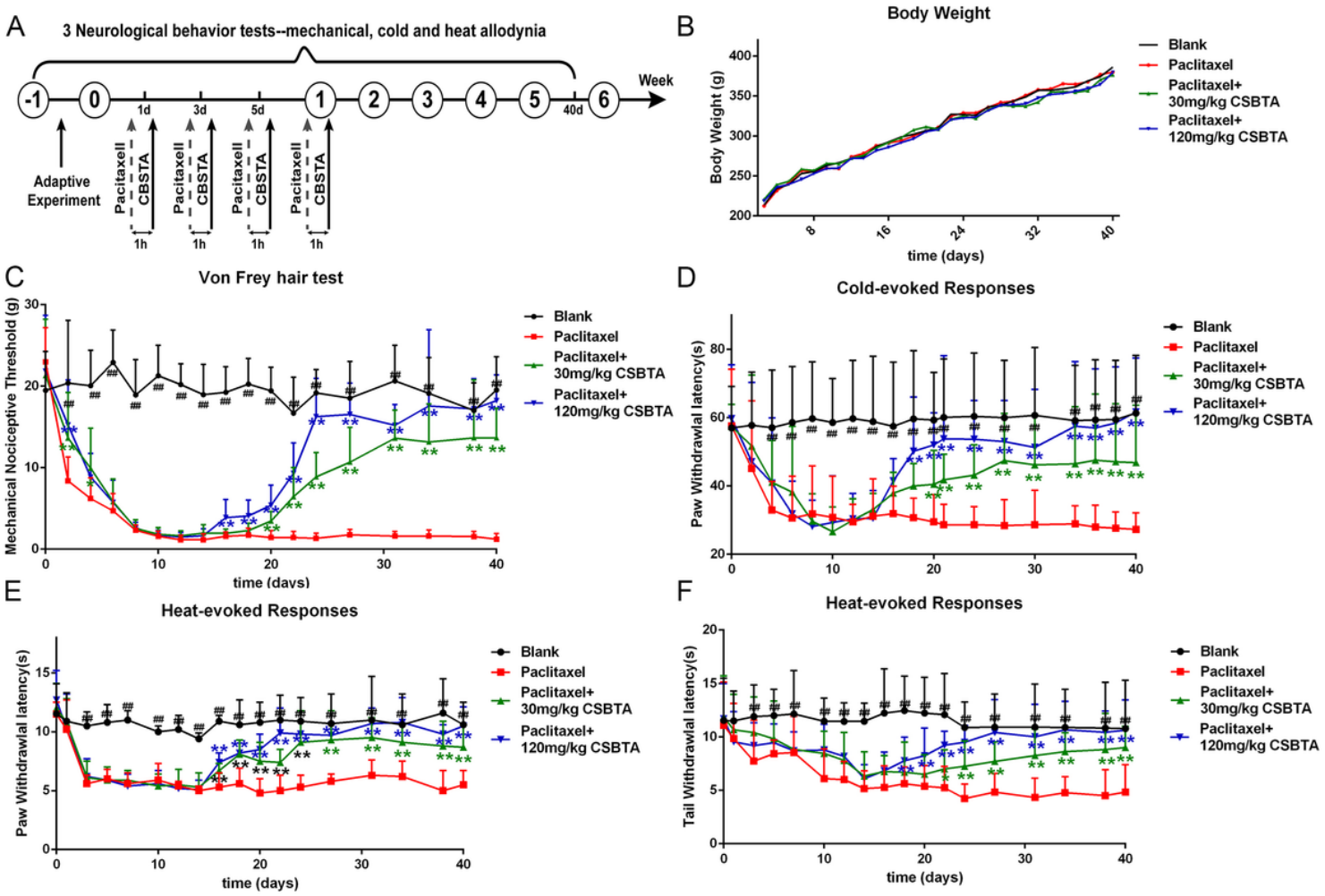
# Tables

**Table 1.** The primers for the quantitative RT -PCR.

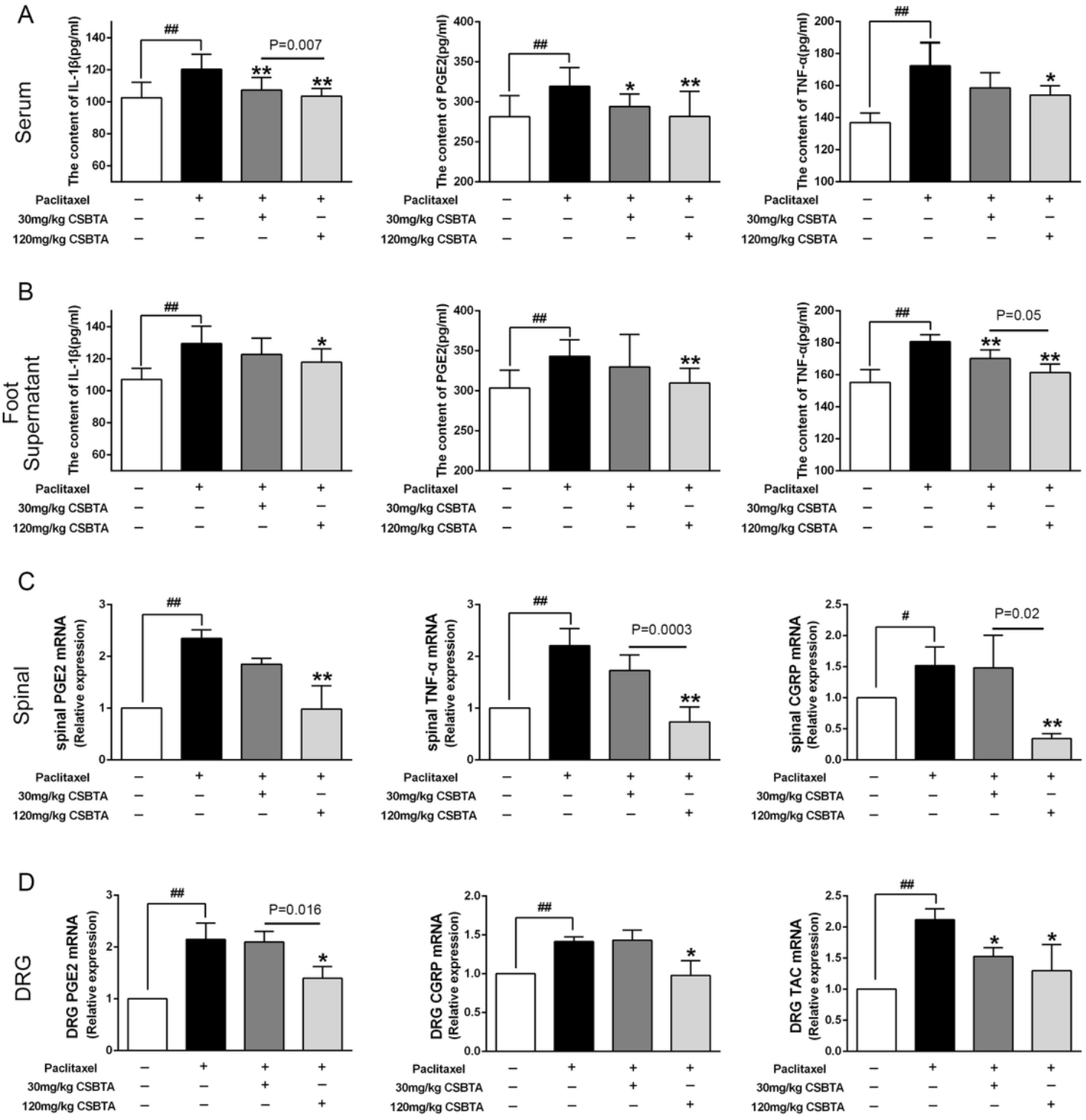
| Gene | Forward Primer | Reverse Primer |
|---|---|---|
| TRPV1 | AGAAGGGGAACCAGGGCAAAG | TCAACGAGGACCCAGGCAACT |
| PKC epsilon | TCTGGAAGCAGCAATAGAGTT | TCATCAAGGTGTTAGGCAAAG |
| Tac1 | CTCACAAAAGGCATAAAACAGATT | TGAATAGATAGTGCGTTACAGGGTT |
| CGRP | GTCCCTCCTCTCCTTTCCAGTT | AGATTCCAGATACCATCCTTTGCC |
| PGE2 | AGGGAGCATACAGCGAAGGTG | TGCGGATTGTCTGGCAGTAGC |
| TNF-α | GCGTGTTCATCCGTTCTCTACC | TACTTCAGCGTCTCGTGTGTTTCT |
| β-actin | ATCATTGCTCCTCCTGAGCG | CGCAGCTCAGTAACAGTCCG |

# Figures




## Figure 1

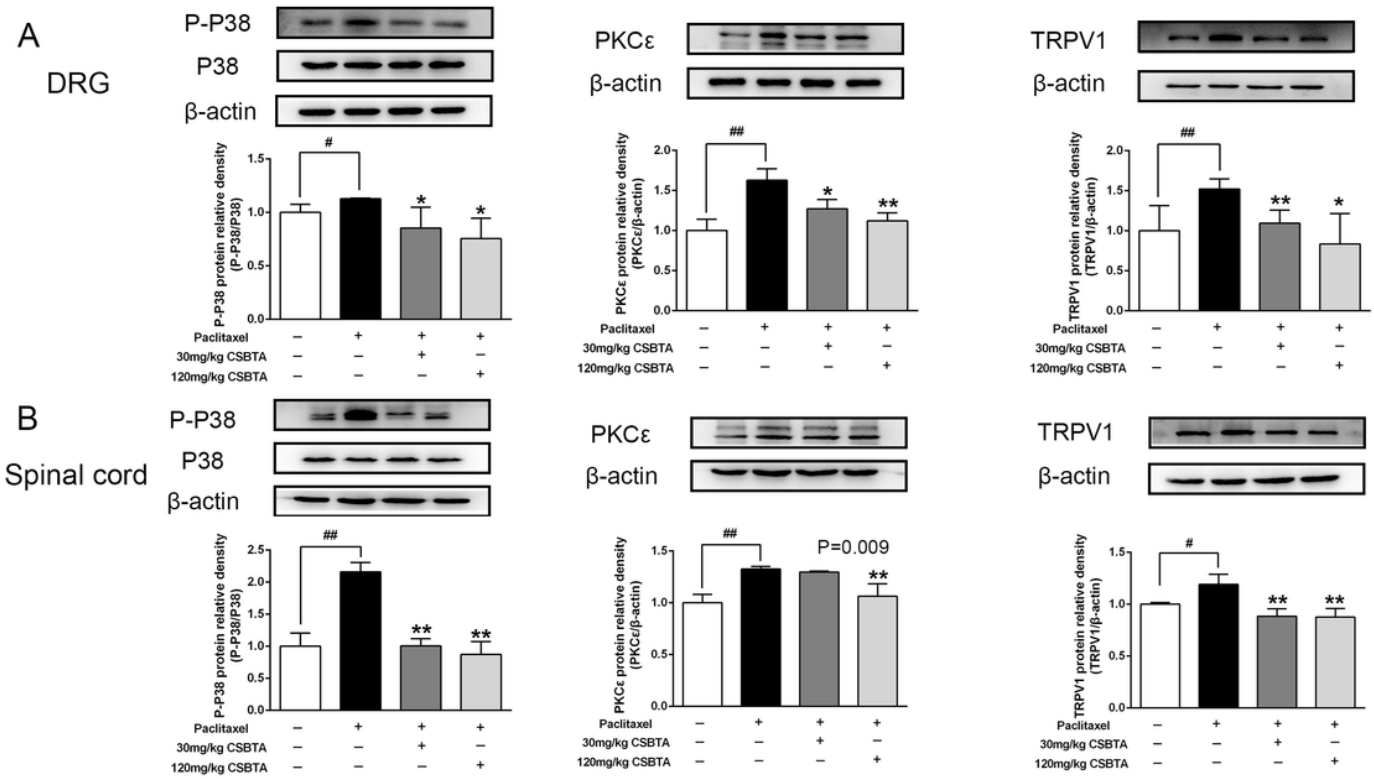
The neuroprotective effects of CSBTA in the PIPN model. (A) In the first week, 2 mg/kg paclitaxel was injected intraperitoneally every other day and vehicle or CSBTA (30mg/kg or 120mg/kg) was administrated by intraperitoneal injection one hour before paclitaxel treatment. (B) The mean body weight growth curve during the experimental period. (C) The Von Frey filament test was used to detect the change of mechanical allodynia in rats. The lower the value, the higher the sensitivity of the animal to mechanical stimulation. (D) Drikold stimulation experiment was used to detect the changes of cold hyperalgesia. The lower the value, the higher the sensitivity of animals to cold. (E, F) Laser heat tingling and tail immersion test were used to detect the sensitivity of rats to thermal stimulation. The results were presented as means ± SD from 8 rats in each group. ##p<0.01, #p<0.05 compared with Blank group; *p<0.05, **p<0.01 compared with Paclitaxel model group.




## Figure 2

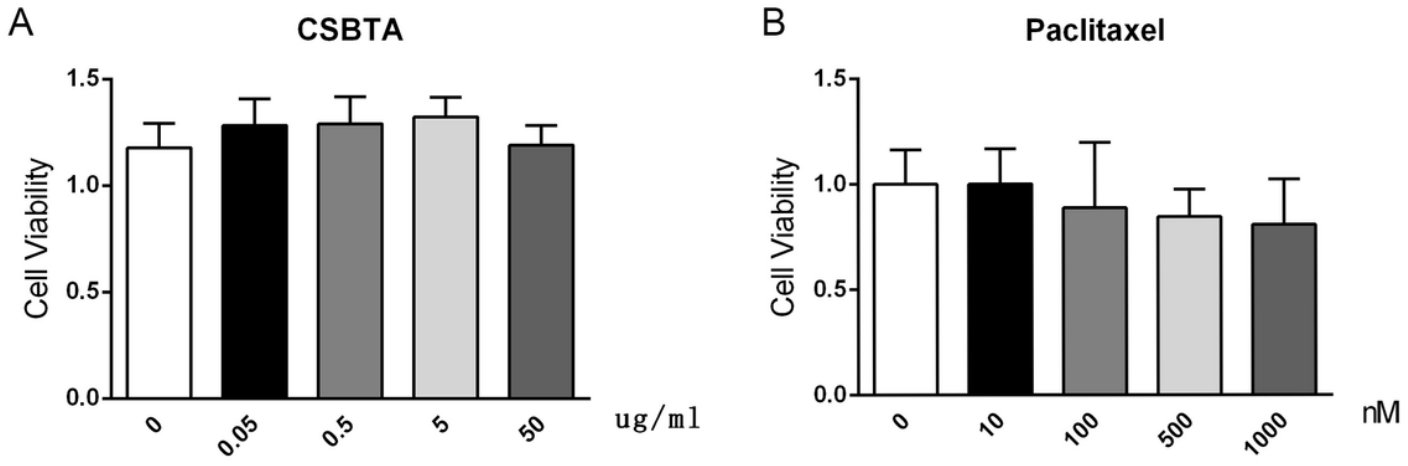
The effects of CSBTA on the expression levels of IL-1β, TNF-α, PGE2, and neuropeptides SP and CGRP in serum and tissues of rats. (A) Serum and (B) plantar skin supernatant were measured by enzyme-linked immunosorbent assay. (C) Spinal and (D) DRG were measured by RT-qPCR. Except for Blank groups, animals from all groups got Paclitaxel (2 mg/kg) from Day1. Rats were pretreated with CSBTA (30 mg/kg or 120 mg/kg) at given doses. The results were presented as means ± SD from 8 rats in each group.

##p<0.01, #p<0.05 compared with vehicle Blank groups; *p<0.05, **p<0.01 compared with Paclitaxel model group.



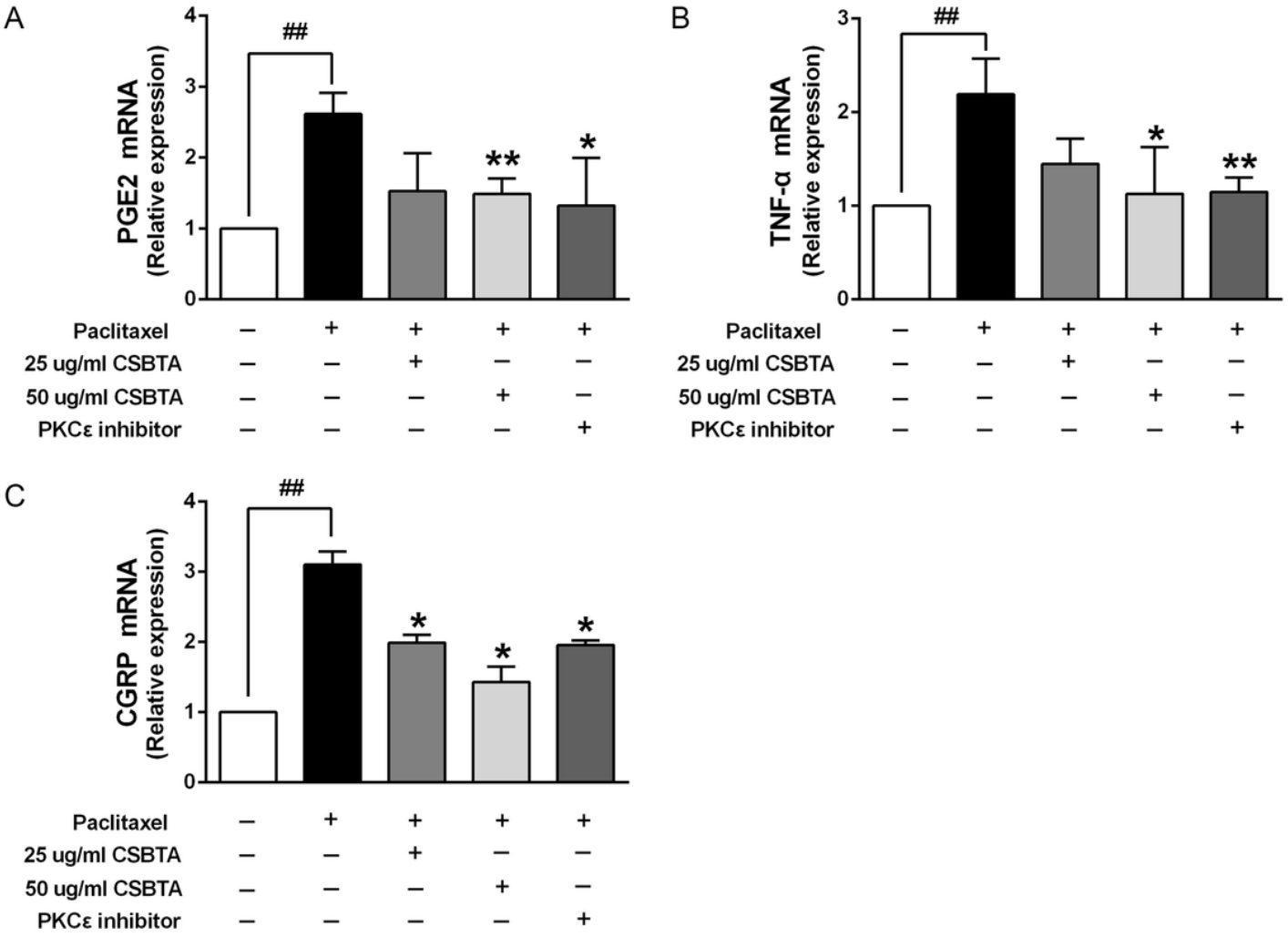

## Figure 3

The effect of CSBTA on the protein expression level of DRG(A), and spinal cord(B), in the paclitaxel-induced CIPN rat model. Except for Blank groups, animals from all groups got Paclitaxel (2 mg/kg) from Day1. Rats were pretreated with CSBTA (30 mg/kg or 120 mg/kg) at given doses. Grey values were processed as the percentage of the optical density (O.D.). Protein levels of p-p38 expressed in tissues were presented as a ratio of O.D. of the corresponding p38. The rest of the protein levels were presented as a ratio of O.D. of the corresponding β-actin. The corresponding quantitative data were present as mean ± SD. n=5, #p<0.05, ##p<0.01 compared to the Blank group, *p<0.05, **p<0.01 compared to the Paclitaxel group.



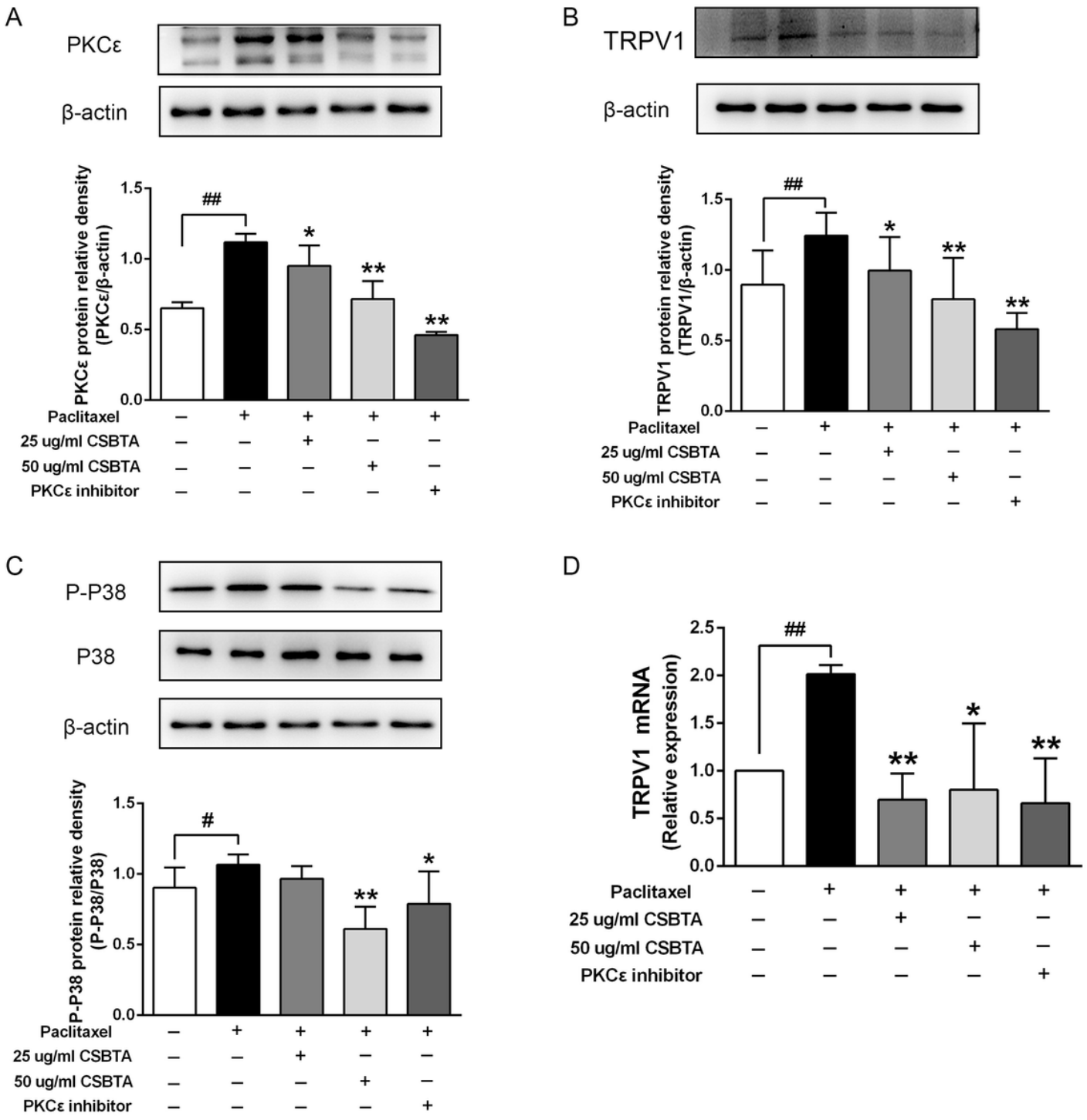

## Figure 4

Cell viability was measured after paclitaxel/CSBTA administration at different concentrations. After three days of growth, the cell state is stable. (A) CSBTA (0, 0.05, 0.5, 5, 50 μg/ml) and (B) paclitaxel (0, 10, 100, 500, 1000 nM) were administered for 5 days, and CCK-8 solution was added to measure the absorbance value at 450 nm. The results were presented as means ± SD, n=3. ##p<0.01, #p<0.05 compared with Blank group; *p<0.05, **p<0.01 compared with Paclitaxel group.




## Figure 5

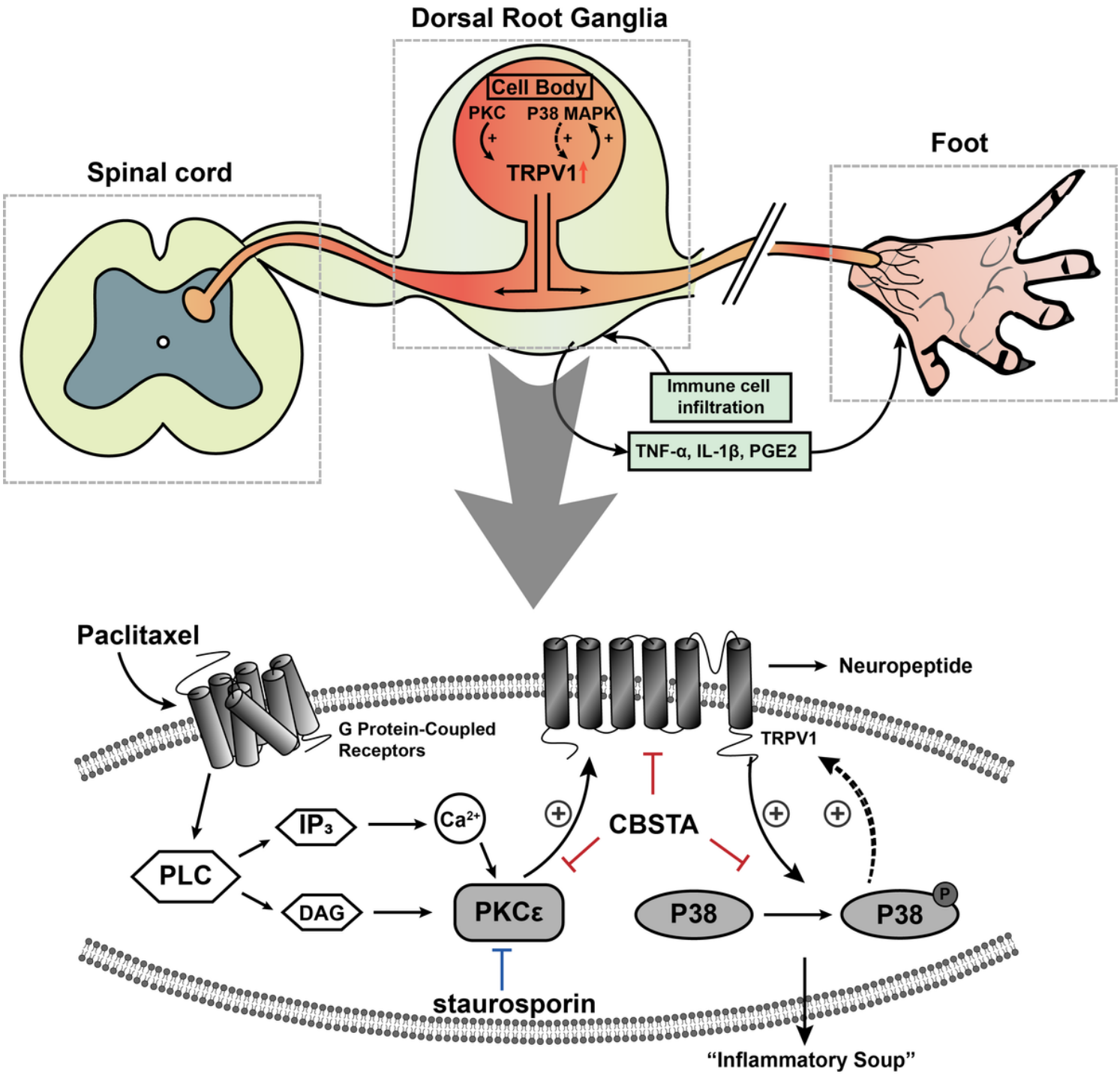
The effect of CSBTA on PGE2 (A), TNF-α (B), and CGRP (C) mRNA levels in paclitaxel-stimulated DRG neurons. After three days of growth, the cell state is stable. DRG neurons were sensitized with paclitaxel (300 nM) and co-incubation with CSBTA (25 μg/ml, 50 μg/ml) and Staurosporine (100 nM) for 5 d. The content was determined by RT-qPCR. The results were presented as means ± SD, n=3. ##p<0.01, #p<0.05 compared with Blank group; *p<0.05, **p<0.01 compared with Paclitaxel group.




## Figure 6

Effect of CSBTA on the protein expression of PKCε (A), TRPV1 (B), and p-p38 (C) and the TRPV1 mRNA level (D) in paclitaxel-stimulated DRG neurons. After three days of growth, the cell state is stable. DRG neurons were sensitised with paclitaxel (300 nM) and co-incubation with CSBTA (25 μg/ml, 50 μg/ml) and Staurosporine (100 nM) for 5d. Protein levels of p-p38 expressed in tissues were presented as a ratio of O.D. of the corresponding p38. The PKCε and TRPV1 protein levels were presented as a ratio of O.D. of

the corresponding β-actin. The corresponding quantitative data were present as mean ± SD. n=5, #p<0.05, ##p<0.01 compared to the Blank group, *p<0.05, **p<0.01 compared to the Paclitaxel group.




## Figure 7

Schematic summary of CSBTA therapeutic effect and mechanism on PIPN.