

Shaoyao Gancao Decoction Ameliorates Paclitaxel-Induced Peripheral Neuropathy via Suppressing TRPV1 and TLR4 Signaling Expression in Rats

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Purpose: Paclitaxel-induced peripheral neuropathy (PIPN) is increasingly becoming one of the most widespread adverse effects in the treatment of cancer patients, and further precipitate neuroinflammation in the nervous system. Interestingly, Shaoyao Gancao Decoction (SGD), a traditional Chinese analgesic prescription, has emerged as a primary adjuvant to chemotherapy in relieving side effects, especially in the case of PIPN. However, the underlying mechanism of SGD functioning in PIPN remains elusive. Accordingly, the current study set out to explore the potential axis implicated in the functioning of SGD in PIPN.

Methods: First, network pharmacology was adopted to predict the role of the transient receptor potential vanilloid type 1 (TRPV1) protein in treating PIPN with SGD. Subsequently, the effects of SGD treatment on mechanical allodynia and thermal hyperalgesia were evaluated in rat PIPN models. Based on the bioinformatics information and current literature, paclitaxel activates toll-like receptor 4 (TLR4) induces the sensitization of TRPV1 mechanistically. Thereafter, TLR4-myeloid-differentiation response gene 88 (MyD88) signaling and TRPV1 expression patterns in dorsal root ganglia (DRGs) were measured by means of Western blotting, qPCR and immunofluorescence.

Results: Initial bioinformatics reared a total of 105 bioactive compounds and 1075 target genes from SGD. In addition, 40 target genes intersected with PIPN were considered as potential therapeutic genes. Based on the network analysis, SGD was found to exert its analgesic effect by reducing the expression of TRPV1. Further experimentation validated that SGD exerted an analgesic effect on thermal hyperalgesia in PIPN models, such that this protective effect was associated with the suppression of TRPV1 and TLR4-MyD88 Signaling over-expression.

Conclusion: Collectively, our findings indicated that SGD ameliorates PIPN by inhibiting the over-expression of TLR4-MyD88 Signaling and TRPV1, and further highlights the use of SGD as a potential alternative treatment for PIPN.

Keywords: paclitaxel-induced peripheral neuropathy, Shaoyao Gancao Decoction, transient receptor potential vanilloid type 1, network pharmacology, paclitaxel, toll-like receptor 4

Introduction

Cancerous malignant tumors are one of the most prevalent causes of disease and morbidity in urban populations, and further accompanied by an ever-increasing incidence.¹ Despite the advent of novel therapies such as immunotherapy and targeted therapies, the use of chemotherapy still remains the gold-standard for most malignancies due to its high efficacy.^{2,3} Paclitaxel, a well-known anti-cancer agent produced from the *Taxus brevifolia*'s needles and bark, is extensively applied all over the world to treat a number of malignancies, including ovarian cancer, non-small cell lung cancer, breast cancer and other cancers.⁴⁻⁸ Paclitaxel stabilizes microtubules and reduces their dynamicity via binding to beta-tubulin, and the resulting

microtubules exhibit resistance to depolymerization, which leads to mitotic arrest and consequently to tumor cell death.^{9,10} Although it is highly effective in blocking the proliferation of cancer cells, peripheral neuropathy is encountered as one of the most severe adverse effects of paclitaxel in a large proportion of patients undergoing chemotherapy.¹¹ These patients often present with persistent changes in perception, such as numbness, tingling sensation, and sharp and burning pains.¹² Unfortunately, to date, there are no effective regimens to successfully tackle paclitaxel-induced peripheral neuropathy.¹³ Moreover, single target drugs have previously been applied to treat PIPN, but to no avail. Accordingly, it is imperative to advance the search for novel therapeutic targets to reduce the plight of PIPN.

Shaoyao Gancao Decoction (SGD), one of the most celebrated Chinese herbal prescriptions, also known as Shakuyaku-Kanzo-to in Japan, is composed of *Radix Paeoniae Alba* (*Paeonia lactiflora* Pall., root) (PA) and *Glycyrrhizae uralensis* (*Glycyrrhiza uralensis* Fisch., root and rhizome, honeyed) (GU) at a ratio of 1:1.^{8,14,15} Historically, it was first reported in the *Treatise on Cold Pathogenic and Miscellaneous Diseases*, a traditional Chinese medical classic focusing on exogenous and internal diseases written by Zhang Zhongjing of the Han Dynasty.¹⁶ Currently, SGD is often prescribed for the treatment of patients with muscle spasm and gynecological disorders in Asia, owing to its analgesic and anti-inflammatory effects.^{17–19} Moreover, there is a plethora of evidence to certify the alleviatory effects of SGD on the pain symptoms associated with PIPN with almost negligible side effects.²⁰ Meanwhile, SGD is well established to exert its effects in accordance with the multi-target and multi-pathways principle. However, almost no researchers have explored the underlying mechanism with regard to SGD alleviatory effect on PIPN.

The transient receptor potential vanilloid type 1 (TRPV1), a heat-activated cation channel with high permeability for Ca²⁺, is primarily expressed in the spinal cord and dorsal root ganglia neurons.^{21–23} Existing evidence suggests that TRPV1 can be activated by a wide range of mechanisms, such as heat, capsaicin, secondary messengers, reactive oxygen and nitrogen species.^{22,24} What is noteworthy is that, activation and sensitization of TRPV1 was previously associated with the paclitaxel treatment-induced enhancement of hyperalgesia.²⁵ Moreover, selective blockade of TRPV1 channel with TRPV1 antagonist (AMG9810) is known to completely reverse paclitaxel-induced thermal hyperalgesia.²⁶

The Toll-like receptor 4 protein (TLR4), a type of pattern-recognition receptor (PRR), is known to confer an indispensable role in the mediation of chronic itch and pain.^{27,28} The initiation and up-regulation of TLR4, combined with its downstream molecule MyD88, have also been previously documented in paclitaxel-related chemotherapy-induced peripheral neuropathy.^{29,30} Moreover, a prior study revealed that inhibiting or knocking out the expression of TLR4 exerts a diminishing effect on injury-induced neuropathy pain.^{29,31} Previous studies have also found that TRPV1 was sensitized via TLR4 after utilizing paclitaxel to activate peripheral sensory neurons.^{32,33} Thus, it could be possible that paclitaxel activates sensory neurons by sensitizing TLR4-MyD88 signaling and TRPV1 channel, inducing nociceptive hypersensitivity and consequently resulting in neuropathic pain. However, there is a lack of reliable evidence to substantiate the treatment functions of SGD on PIPN, and the relevant mechanism of the TRPV1 and TLR4-MyD88 signaling. Accordingly, the current study set sought to explore SGD's analgesic functions and the underlying biomolecular mechanism in regard to PIPN, with the help of network analyses, animal models and biomolecular experiments (Figure 1).

Materials and Methods

Network Target Analysis

Searching Bioactive Ingredients in SGD

The traditional Chinese medicine systems pharmacology (TCMSP) database (<http://tcmspw.com/>) was adopted to retrieve and collect bioactive elements of SGD.³⁴ Ingredients were then filtered using the following selection criteria: oral bioavailability (OB) threshold $\geq 30\%$ and drug-likeness (DL) threshold ≥ 0.18 .

Protein Target Genes in Databases

The SwissTargetPrediction database (<http://www.swisstargetprediction.ch/>) and TCMSP database (<http://tcmspw.com/>) were utilized to screen SGD protein targets. Related target genes of PIPN were additionally obtained from the OMIM therapeutic database (<http://www.ncbi.nlm.nih.gov/omim/>). In addition, pain-related target genes were collected from the GeneMANIA database (<https://genemania.org/>), using the following keywords: paclitaxel-induced peripheral neuropathy, paclitaxel-induced peripheral neuropathic pain and paclitaxel-induced neuropathic pain. The UniProt

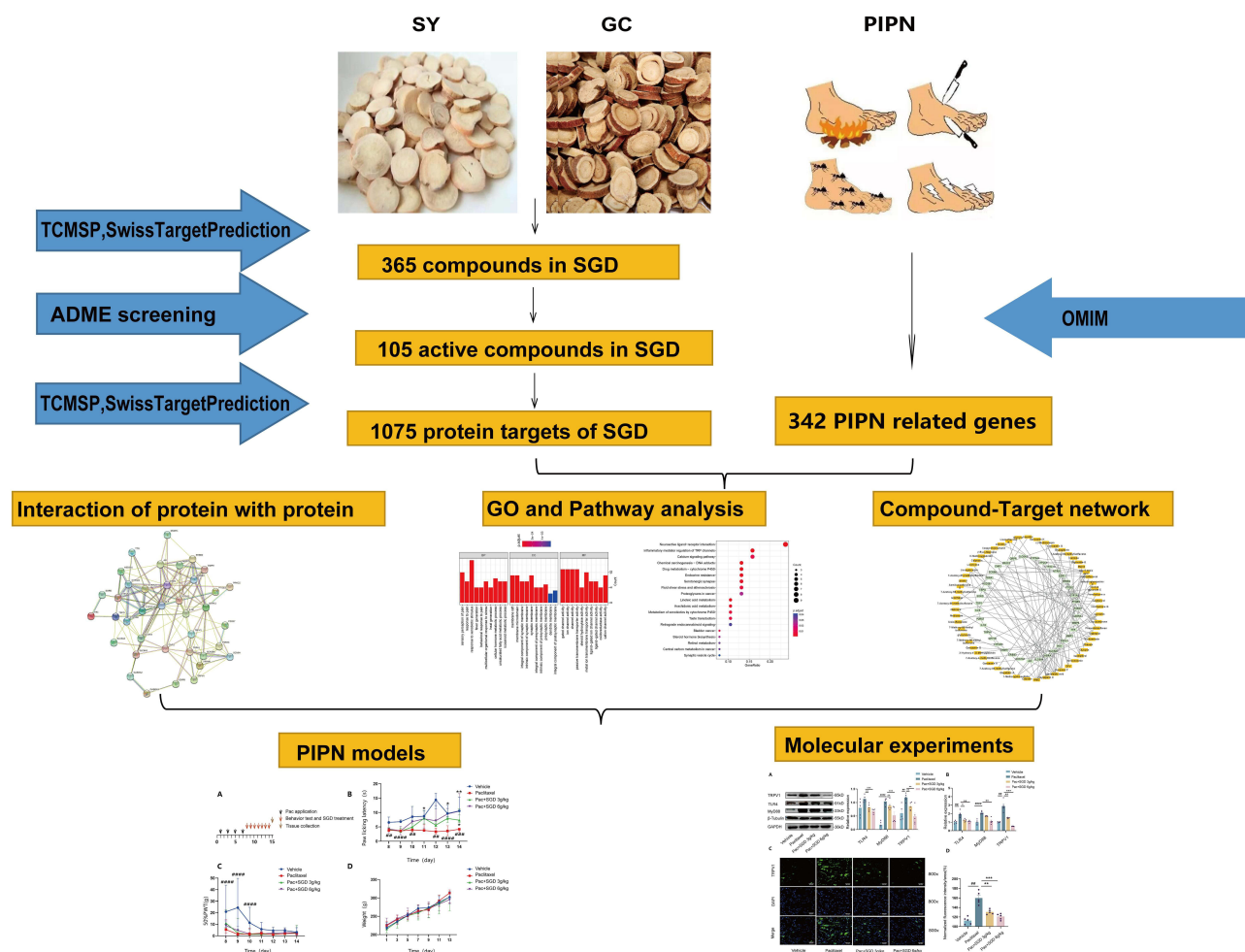


Figure 1 The workflow of the current study.

Abbreviations: SY, Shaoyao; GC, Gancao; PIPN, paclitaxel-induced peripheral neuropathy; SGD, Shaoyao Gancao Decoction; TCMSP, traditional Chinese medicine systems pharmacology database; ADME, absorption, distribution, metabolism, and excretion; OMIM, Online Mendelian Inheritance in Man.

database (<https://www.uniprot.org/>) was then adopted to standardize all the aforementioned target names. Intersection genes (ie repetitive target genes) regarding SGD treatment for PIPN were finally obtained using the Venny 2.1 tool (<https://bioinfo.gp.cnb.csic.es/tools/venny/>). Venn diagram was established by Microsoft Office PowerPoint.

Construction of Protein–Protein Interaction (PPI) Network

In order to establish a protein–protein interaction (PPI) network, the STRING10.5 online database (<https://string-db.org/cgi/help.pl?UserId=PWTj1MTAhQKH&sessionId=8w1BKy7kNz51>) was utilized to analyze PIPN-SGD-related target proteins. In the PPI network map, each node symbolized a protein gene and each edge denoted a functional link between potential target genes. The Cytoscape software was then adopted to analyze the network after importing target genes into it.

Establishment of Compound-Target Network

To explore the pharmacological mechanisms, a compound-target network was constructed between the 40 potential target genes and bioactive ingredients of SGD using the Cytoscape 3.7.2 software. Each node symbolized the target genes and compounds, and similarly, lines denoted the interactive association between the target genes and compounds in the compound-target network. Key compounds and target genes were subsequently selected using the network topology parameters.

GO and KEGG Pathway Enrichment Analysis

The R package (org.Hs.eg.db, version 3.8) was utilized to further explore the Gene Ontology (GO) and KEGG (Kyoto Encyclopedia of Genes and Genomes) databases. Terms presented with thresholds Count ≥ 2 and Expression Analysis Systematic Explorer (EASE) scores ≤ 0.05 were selected for further functional clustering.

Next, in order to further investigate the relative signaling pathway regarding TRPV1 and the target genes of pain, the GeneMANIA database was utilized for prediction. Additionally, KEGG pathway enrichment analysis was also performed.

Experiment for Verification

Chemical Procurement

Paclitaxel was procured from Hospira Australia Pty. Ltd. (Australia). Saline (0.9% NaCl injectable solution) was purchased from Kelun Pharm Co. Ltd. (Chengdu, China). Shaoyao (*Paeoniae Radix Alba*, the root of *Paeonia lactiflora* Pall.) and Gancao (*Glycyrrhizae Radix et Rhizoma*, the root and rhizome of *Glycyrrhiza uralensis* Fisch.) were obtained from the Xiangya Hospital of Central South University (Changsha, China). Prof. SY Hu from the department of Chinese Herbal Medicine of Central South University carried out the authentication of the acquired crude herbs. The voucher specimens (No. 21040908 and No. 21041608) were deposited at the Ethnopharmacology Laboratory of Xiangya Hospital.

Ethics Concerns

The current study was approved by the Medical Ethics Committee of Central South University (approval no. 2021sydw0146), and in accordance with guidance from Central South University for the use and care of animals. Extensive efforts were undertaken to minimize both the number and suffering from the experimental animals.

Animal Usage

Total 20 male Sprague–Dawley rats (5–8 weeks, weighing 270–300 g) were acquired from the Department of Laboratory Animal Research at Central South University. Every 3 rats were housed in laminar flow cages and sustained in conditions of 50%–60% relative humidity, 22 \pm 2°C temperature, under a 12-h light/12-h dark cycle as previously described.⁸ The rats were allowed *ad libitum* access to a standard chow diet and tap water. The rats were housed in the aforementioned cages for at least 3 days for acclimatization prior to experimentation.

PIP-N Rat Model Establishment

Firstly, 6 mg/mL concentrated paclitaxel (Hospira Australia Pty. Ltd, Australia) was diluted by a concentration of 1 mg/mL with the addition of 0.9% saline. Subsequently, the rats were intraperitoneally administered diluted paclitaxel (a dosage of 2 mg/kg) every other day on days 1, 3, 5, and 7. Meanwhile, rats in the control group received only saline treatment of the same volumes.

Drug Administration

SGD preparation was carried out in accordance with previously published literature.^{8,35} Briefly, Baishao (100 g) and Gancao (100 g) were blended and distilled twice in water (at a ratio of 1:10, g/mL) for 1 h. The combination included the above two filtrates, and the concentration of SGD was determined to be 1g/mL. The decoction was stored at 4°C for subsequent oral gavage to rats. A total of 4 groups of rats (n = 5 for each treatment) were established using the random number table method. Two rat groups were administered SGD gavage (dosages of 3g/kg and 6g/kg) from day 8 to day 14, and for the control group and paclitaxel group, rats were allowed access to distilled water as control.

Mechanical Allodynia

The above-mentioned rats were habituated in acrylic transparent chambers one by one on a mesh floor, for at least 15 minutes prior to any experimentation until they were adapted to the new environment and stopped cage exploration. Next, to measure the mechanical allodynia, the mid-plantar surface of the hind paws of rats was pricked with von Frey filaments (Stoelting, IL, USA), with appropriate power bending the filament for 6–8 s slightly. Active responses included the following: a sharp withdrawal of the paw, licking and immediate flinching after stimulation.³⁶ In order to calculate the

threshold, the up-down paradigm was adopted, and based on the nonparametric Dixon test, 50% paw withdrawal threshold (PWT) was measured and recorded.³⁶ During the whole behavior test, the experimenter was blinded to all of the rat groupings.

Thermal Hyperalgesia

The above-mentioned rats were habituated in acrylic transparent chambers one by one for at least 15 minutes prior to any experimentation. An Incremental Hot Cold Plate (IITC Life Science, Woodland Hills, CA, USA) was utilized to assess thermal hyperalgesia. Briefly, the rats were placed on the surface of the hot plate (heated to a temperature of $55 \pm 2^\circ\text{C}$) one at a time. Positive responses included the following: jumping or licking the hind-paw. A 20-s cutoff threshold was set up to prevent tissue damage. A significant drop in paw licking latency was regarded as heat hyperalgesia. During the whole behavior test, the experimenter was blinded to all of the rat groupings. Each rat's thermal latency was measured 3 times to obtain the mean value.

Tissue Collection

After the completion of behavioral testing, the rats were anesthetized with the administration of sodium pentobarbital (Sigma-Aldrich Co, St Louis, Mo, USA). Next, the rats were intracardially perfused with 4% paraformaldehyde. DRGs of Left and right L4-6 and segments of L4-6 spinal cord were immediately excised and stored in liquid nitrogen. The obtained tissue samples were maintained at -80°C prior to further experimentation. Later, the tissues were cryoprotected in 20% sucrose and 30% sucrose at 4°C for at least 48 h.

Quantitative RT-PCR Analysis

The TRIzol reagent (Invitrogen, Waltham, MA) was adopted for the extraction of total RNA content, and PrimeScript™ kits were (TaKaRa Bio Inc, Otsu, Japan) utilized for the synthesis of cDNA, in accordance with the manufacturer's protocols. A SYBR Green fluorescence-based assay (TaKaRa Bio Inc, Otsu, Japan) was adopted to perform qRT-PCR on a ViiA™ 7 RT-PCR system (Applied Biosystems, Carlsbad, CA). The primers for real-time PCR were as follows: TRPV1: forward: CCAGTCAAGCCCCACATCTT; reverse: CCAGCTCCTGGCAGTTACTC. GAPDH: forward: TGTGGGCATCAATGGATTG; reverse: ACACCATGATGGGGGGGGATCAAT. TLR4: forward: CCGCTCTG GCATCATCTTCA; reverse: TCCCACTCGAGGTAGGTGTT. MyD88: forward: TGTCTCCCCTGACATGCCTA; reverse: CTGGGGGCGGAATGTTTTTGGAPDH. GAPDH was adopted as the reference control. The relative mRNA expressions were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method.

Western Blot Assay

SGD and spinal cord samples were centrifuged and immersed in RIPA buffer (P0013B, Beyotime, Shanghai, China) containing protease inhibitor (Thermo Fisher Scientific, Waltham, MA, United States) for the extraction of total protein content. The preparations were collected and stored at -80°C . The equal amounts of protein (10–50 μg) were separated by means of 10% SDS-PAGE, and then transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA). The membranes were subsequently blocked with 5% skim milk for 2 h at 37°C , and incubated with primary antibodies against TRPV1 (ab6166, Abcam, Cambridge, UK), TLR4 (66350, abclonal, Wuhan, China) and MyD88 (ab219413, Abcam, Cambridge, UK) overnight at 4°C . The following day, immunoreactive bands were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (#7074 CST, MA, USA) for 30 minutes at room temperature. The ChemiDocXRS+ System (Bio-Rad, Hercules, CA) was utilized for imaging the bands, which were analyzed with the ImageJ software (NIH). The relative expression was calculated as the ratio of the intensity of the gene of interest to that of GAPDH (abs132004, absin, China) and β -Tubulin (#2148S CST, MA, USA).

Immunofluorescence Staining

A frozen microtome (Thermo NX50, MA, USA) was utilized to cut the spinal cord cross sections (thickness of 20 μm), and these sections were transferred onto gelatin-coated glass slides to prepare immunofluorescence staining. Following pre-incubation with 10% normal goat serum and 0.3% Triton X-100 in PBS for 1 h at room temperature, a primary antibody (rabbit anti-TRPV1, ab203103, Abcam, Cambridge, UK) was applied for 18 h at 4°C . Subsequently, the sections were rinsed with PBS thrice, and then incubated with a secondary antibody conjugated to Alexa fluor 488 (488

goat anti-rabbit antibody ab150077, Abcam, Cambridge, UK) for 1 h at 37 °C. Afterwards, the nuclei were stained with DAPI and visualized under a Leica DM5000 B microscope (Leica Microsystems, Buffalo Grove, IL, USA).

Statistical Analysis

Statistical analyses were performed using the GraphPad Prism 7.0 software. Non-normal data were presented as median and range (min–max). Normal data were presented in the form of mean \pm SD. At first, Mauchly's test of sphericity and QQ plots together with Shapiro–Wilk test were applied to assess data distribution. Behavioral data were analyzed with two-way ANOVA followed by Tukey's post hoc test. Biomolecular experiment data were analyzed by Student's *t*-test or Mann–Whitney *U*-test accordingly. A value of $p < 0.05$ was regarded as statistically significant.

Results

Network Target Analysis

Identification of the Active Compounds in SGD

A total of 365 compounds were obtained from the TCMSP and SwissTargetPrediction database, which included 85 compounds in BS and 280 compounds in GC after excluding duplicates. Among the 85 ingredients in BS, 13 active compounds met the selection criteria of OB $\geq 30\%$ and DL ≥ 0.18 . Meanwhile, among the 280 compounds in GC, 92 active compounds met the selection criteria of OB $\geq 30\%$ and DL ≥ 0.18 . Following ADME screening, altogether 105 components were selected as candidate bioactive components for further analyses.

Target Genes and PPI Network Map

A total of 342 PIPN target genes were obtained from the OMIM databases. Similarly, following analyses of the 105 active ingredients of SGD from the TCMSP and SwissTargetPrediction database, 1435 target genes were acquired. Subsequently, 1075 target genes were screened after duplicate removal. Next, a sum of 40 potential targets (Figure 2A, Table 1) were found at the intersection of PIPN target genes and SGD target genes using the Venny 2.1 software. Thereafter, a protein–protein interaction network was constructed (Figure 2B) to explore the relationship between the 342 PIPN target genes and 1075 target genes, which revealed that six genes are located in the core area. These genes include albumin (ALB), estrogen receptor (ESR1), interleukin-1 beta (IL1B), transient receptor potential cation channel sub-family V member 1 (TRPV1), prostaglandin E synthase 2 (PTGS2), as well as the high-affinity nerve growth factor receptor (NTRK1) gene.

Compound-Target Network and Analysis

The 40 intersected genes and bioactive compounds were carried into the Cytoscape software (version 3.7.2) to acquire a compound-target network of SGD on PIPN (Figure 2C), which included 98 nodes and 129 edges. A single compound of SGD may interrelate with multiple targets, and a single target may relate to multiple compounds. There were a total of 14 high-degree target genes which were correlated with multiple compounds (degree >4) among the potential target genes, namely, ERBB2, CYP1A2, AR, ESR1, GABRA2, GABRA3, HK1, MMP2, MMP9, P2RX3, PTGES, PTGS2, SLC6A2, SLC6A4, TTR and TRPV1. In addition, among the 105 bioactive compounds, 4 high-degree compounds were found to be localized at the core position (degree >4), which included icos-5-enoic acid, gadelaic acid, glyasperin C and kanzonol F.

GO and KEGG Pathway Enrichment Analysis

The R package for statistical computing was utilized to clarify the biological processes, cell composition and molecular function of the 40 intersected target proteins (Table 1). Subsequent results showed that there were 386 biological processes (BP), 40 cellular components (CC), and 84 molecular functions (MF) terms. The top 10 categories enriched from BP, CC, and MF are shown in Figure 3A, which implied that SGD may alleviate PIPN through sensory perception of pain, membrane raft, and gated channel activity.

KEGG pathway enrichment analysis was performed to further investigate the potential target genes and significant pathways. As shown in Figure 3B, 20 pathways correlated with 40 protein targets were enriched, including inflammatory mediator regulation of TRP channels, neuroactive ligand–receptor interaction, calcium signaling pathway, chemical

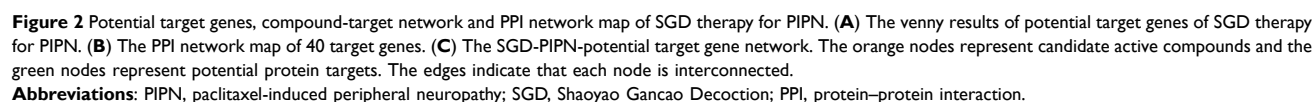


Table 1 40 Potential Target Gene of SGD Treatment for PIPN

No.	Target	Symbol	Entrez ID
1	Albumin	ALB	P02768
2	Androgen receptor	AR	P10275
3	Cannabinoid receptor 1	CNR1	P21554
4	Cytochrome P450 1A2	CYP1A2	P05177
5	Cytochrome P450 2C19	CYP2C19	P33261
6	Cytochrome P450 2C9	CYP2C9	P11712
7	Cytochrome P450 2D6	CYP2D6	P10635
8	Cytochrome P450 3A4	CYP3A4	P08684
9	DNA	DNMT1	P26358
10	Receptor tyrosine-protein kinase erbB-2	ERBB2	P04626
11	Receptor tyrosine-protein kinase erbB-3	ERBB3	P21860
12	Estrogen receptor	ESR1	P03372
13	Gamma-aminobutyric acid receptor subunit alpha-2	GABRA2	P47869
14	Gamma-aminobutyric acid receptor subunit alpha-3	GABRA3	P34903
15	Gastrin-releasing peptide receptor	GRPR	P30550
16	Hexokinase-I	HK1	P19367
17	3 beta-hydroxysteroid dehydrogenase/Delta 5->4-isomerase type I	HSD3B1	P14060
18	Interleukin-1 beta	IL1B	P01584
19	72 kDa type IV collagenase	MMP2	P08253
20	Matrix metalloproteinase-9	MMP9	P14780
21	Neuropeptide Y receptor type 1	NPY1R	P25929
22	High affinity nerve growth factor receptor	NTRK1	P04629
23	P2X purinoceptor 3	P2RX3	P56373
24	P2X purinoceptor 7	P2RX7	Q99572
25	Protein kinase C zeta type	PRKCZ	Q05513
26	Prostaglandin E synthase	PTGES	O14684
27	Prostaglandin E synthase 2	PTGS2	Q9H7Z7
28	Sodium channel protein type 10 subunit alpha	SCN10A	Q9Y5Y9
29	Sodium channel protein type 9 subunit alpha	SCN9A	Q15858
30	Sodium-dependent noradrenaline transporter	SLC6A2	P23975
31	Sodium-dependent dopamine transporter	SLC6A3	Q01959
32	Sodium-dependent serotonin transporter	SLC6A4	P31645
33	Sorbitol dehydrogenase	SORD	Q00796
34	Tyrosyl-DNA phosphodiesterase 1	TDPI	Q9NUW8
35	Transient receptor potential cation channel subfamily A member 1	TRPA1	O75762
36	Transient receptor potential cation channel subfamily M member 8	TRPM8	Q7Z2W7
37	Transient receptor potential cation channel subfamily V member 1	TRPV1	Q8NER1
38	Transient receptor potential cation channel subfamily V member 4	TRPV4	Q9HBA0
39	Translocator protein	TSPO	P30536
40	Transthyretin	TTR	P02766

carcinogenesis – DNA adducts and so on. The key translational implications indicated that the active ingredients of SGD may be useful in the prevention and treatment of PIPN via acting on these signaling pathways.

An additional KEGG pathway enrichment assay was carried out to further explore the relationship between TRPV1 and the target genes of pain ([Supplementary Table 1](#)). Among the 95 pathways, TLRs signaling pathway was found to be enriched with a relatively high gene count and P value.

Experiment for Verification

Shaoyao-Gancao Decoction Attenuated Thermal Hyperalgesia in PIPN Model

Paclitaxel treated rat models were first established to investigate the functional relationship between Shaoyao-gancao decoction and PIPN, following the protocols illustrated in [Figure 4A](#). Rats were intraperitoneally administered with

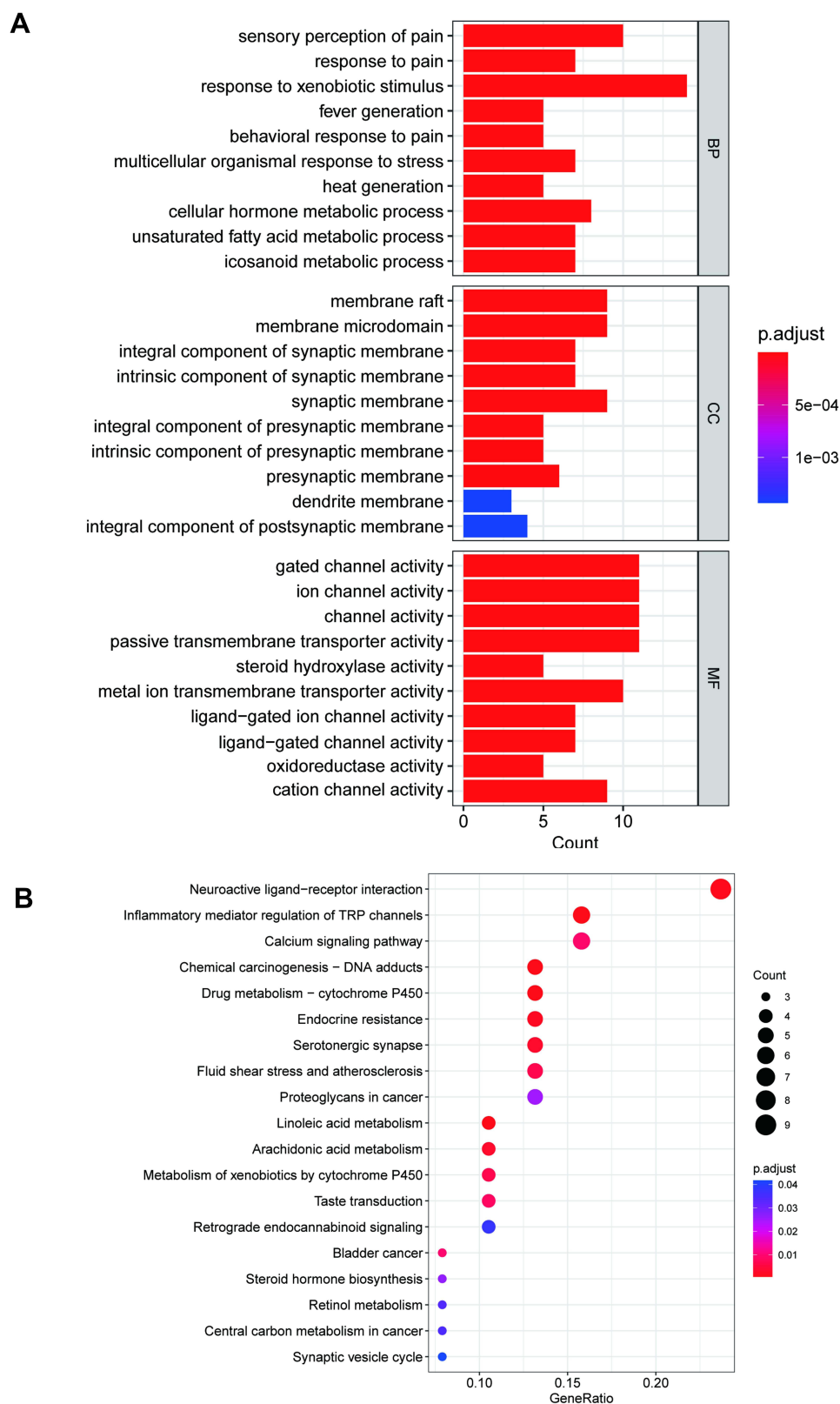


Figure 3 The 10 most significance genes ontology (A) and 20 most significance of pathway enrichment (B) analysis of therapy target genes of SGD on PIPN. **Abbreviations:** PIPN, paclitaxel-induced peripheral neuropathy; SGD, Shaoyao Gancao Decoction.

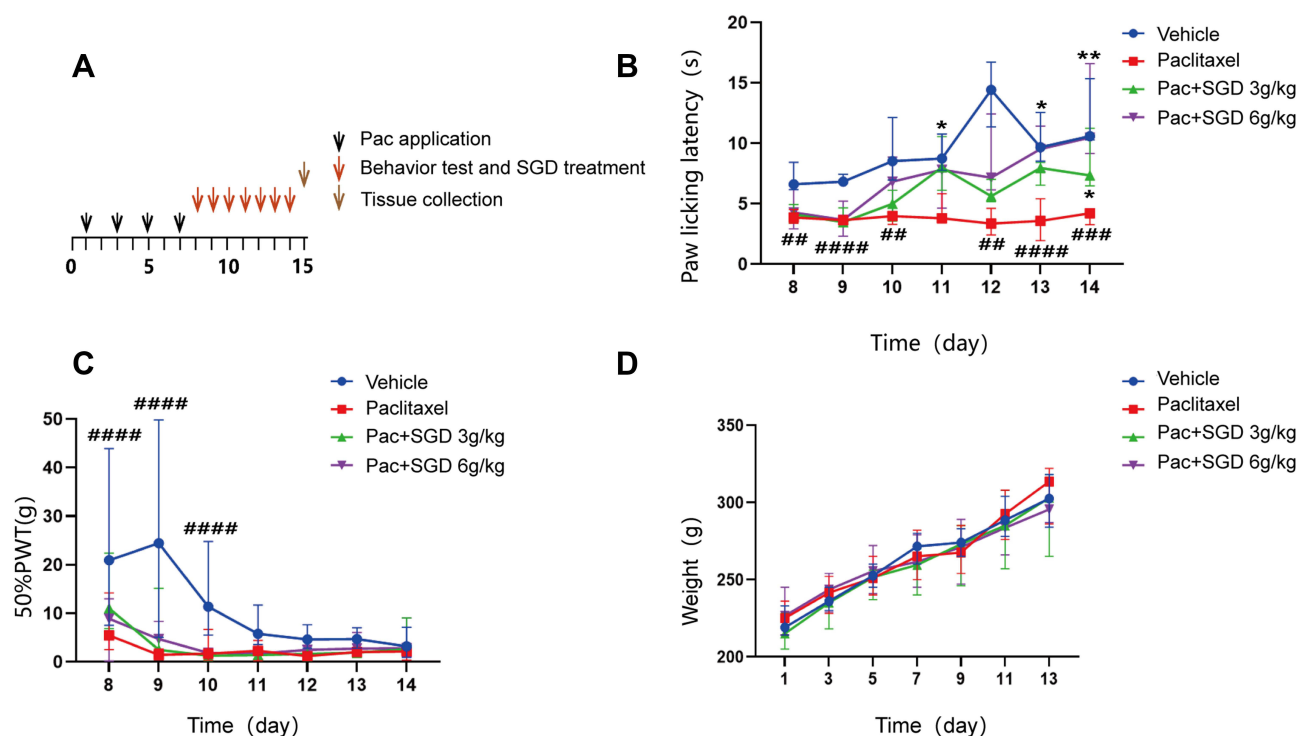


Figure 4 SGD attenuates thermal hyperalgesia in a PIPN model. **(A)** Experiment design for the construction of PIPN model and SGD treatment. **(B)** Reduction of paclitaxel-induced thermal hyperalgesia by administrating SGD for 7 consequent days. **(C)** Effects of SGD treatment on mechanical allodynia of PIPN rats. **(D)** Effects of paclitaxel injection and SGD treatment on body weight of rat models. Data were expressed as median and range (min - max). ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$ versus the vehicle group. * $p < 0.05$, ** $p < 0.01$ versus the paclitaxel group. $n = 5$ rats/group.

Abbreviations: PIPN, paclitaxel-induced peripheral neuropathy; SGD, Shaoyao Gancao Decoction.

paclitaxel (at a dosage of 2 mg/kg for every two days) or vehicle (saline, same volumes and intervals as paclitaxel-injected rats). Prior to any Shaoyao-gancao treatments, the paclitaxel-treated rats exhibited a significant drop in the paw licking latency on the hot plate test ($p < 0.05$) and paw withdrawal threshold based on the Von Frey test ($p < 0.05$), relative to the vehicle-treated rats (Figure 4B and C). These findings substantiated the significant development of peripheral neuropathic hypersensitivity in paclitaxel-induced rat models.

Different concentrations of Shaoyao-gancao decoction were administered to the modelled rats starting from day 8, precisely, the day after the last injection of paclitaxel. SGD elicited significant and persistent relief from thermal hyperalgesia from day 8 compared to that produced by single paclitaxel treatment. As for the SGD 6g/kg group, the paw licking latency was increased from 3.59 ± 0.36 s at day 9 to 9.37 ± 0.77 s at day 14, compared with the SGD 3g/kg group, while the extent of increase was much lower relative to the high concentration of SGD group, suggesting that higher concentration of SGD exerted a more profound effect of relief from thermal hyperalgesia. However, the SGD's effect of anti-allodynia was not mechanically observed until the end of the observation time after applying SGD for a week. Moreover, compared with the vehicle group, there was no change in the body weight of rats undergoing paclitaxel treatment (Figure 4D), and consecutive SGD administration exerted no influence on the body weight of rats either.

SGD Reversed the Up-Regulation of TLR4, MyD88 and TRPV1 in DRGs and Spinal Cord of PINP

The top three pathways in the KEGG analysis, namely, neuroactive ligand–receptor interaction, inflammatory mediator regulation of TRP channels, and calcium signaling pathway, are all associated with TRPV1. In addition, Yukako Kamata et al provided convincing evidence that increased expression levels of TRPV1 channel serve as a key factor in regulating paclitaxel-treated rodents.³⁷ Additional KEGG analysis regarding TRPV1 and the target genes of pain revealed the involvement of the toll-like receptor signaling pathway. Moreover, Yan Li et al have previously demonstrated that for PIPN rats, the expression levels of TLR4 and its downstream signaling MyD88 and TRPV1 are both up-regulated in

DRGs and spinal cord tissues.^{30,33} Furthermore, the peripheral neuropathy of PIPN rats is known to be reduced following blockade of TLR4-MyD88 signaling.³¹ Accordingly, we speculated whether SGD relieved PIPN by means of augmenting TRPV1 and TLR4-MyD88 signaling.

We further explored the molecular mechanisms of SGD's pain-relief function in paclitaxel-induced peripheral neuropathic pain. First, we collected the L4-6 DRGs of rats, given the fact that paclitaxel accumulates in the DRGs of animal models because DRGs lack an effective blood-brain barrier.³⁸ The results of Western blot and Quantitative Real-time PCR demonstrated the presence of increased expressions of TLR4, MyD88 and TRPV1 at the protein and mRNA levels (Figure 5A and B). These findings validated that TLR4, MyD88 and TRPV1 were all up-regulated in L4-6 DRGs following intraperitoneal paclitaxel administration in rats model. On the other hand, regular administration of SGD for one week exerted a counter effect on the aforementioned over-expression, wherein the SGD 6g/kg group exhibited better reversal effects compared to the SGD 3g/kg group.

Existing literature further indicates that chemotherapies can also bring about the activation of astrocyte and microglia in the spinal cord dorsal horn.³⁹⁻⁴² Consequently, immunofluorescence was carried out to observe the mean fluorescence intensity of TRPV1 immune positive (TRPV1+) neurons in the spinal cord. Subsequent results demonstrated that the TRPV1 protein was primarily expressed in the cytoplasm and cell membrane, while there was almost no distribution in the nucleus. As shown in Figure 5C and D, the mean fluorescence intensity of neurons was significantly extended in the

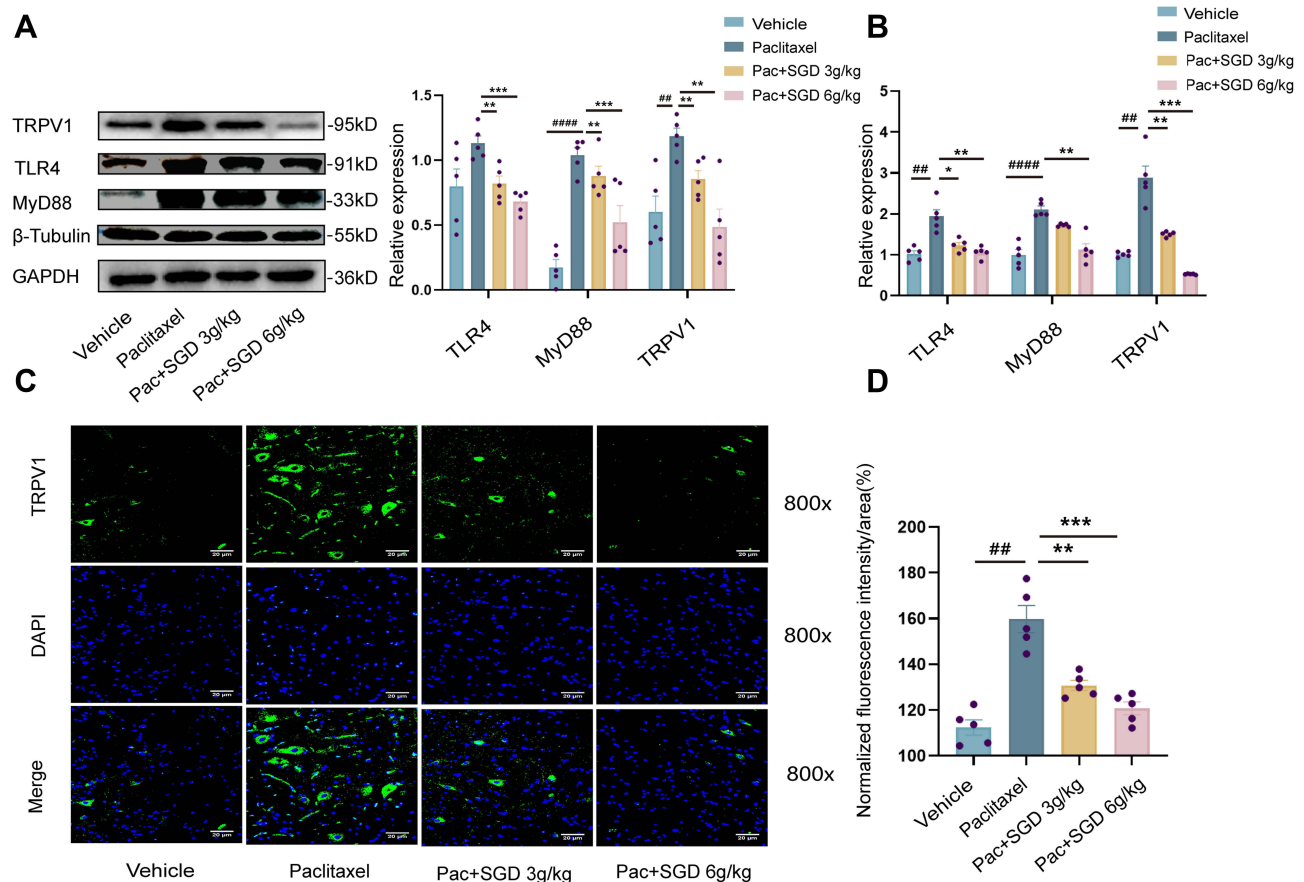


Figure 5 SGD reduced the over-expression of TLR4, MyD88 and TRPV1 in dorsal root ganglion (DRG) neurons or spinal cord from PIPN rats. **(A)** Proteins expression after repeat SGD treatment in PIPN model. The right bar graph shows the proteins expression normalized to GAPDH or β -tubulin. Representative protein images are presented on the left side. **(B)** mRNA expression after repeat SGD treatment in PIPN model. **(C)** Representative immunofluorescence image of TRPV1 and DAPI in vehicle, paclitaxel, paclitaxel + SGD 3g/kg, paclitaxel + SGD 6g/kg. The mean fluorescence intensity **(D)** was also measured. Data were expressed as mean \pm SD. ## $p < 0.01$, ##### $p < 0.0001$ versus the vehicle group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the paclitaxel group. $n = 5$ rats/group.

Abbreviations: PIPN, Paclitaxel-induced peripheral neuropathy; SGD, Shaoyao Gancuo Decoction; TRPV1, transient receptor potential vanilloid type 1; TLR4, toll-like receptor 4; MyD88, myeloid-differentiation response gene 88; DRG, dorsal root ganglia; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DAPI, 4',6-diamidino-2-phenylindole.

paclitaxel-treated group. Importantly, the over-expression of TRPV1 induced by paclitaxel in spinal cord neurons exhibited a deficit response following oral administration of SGD (from 159.74 ± 11.96 to 130.71 ± 4.56 , $P < 0.01$). Furthermore, the mean fluorescent intensity of TRPV1+ neurons showed a significant decrease in SGD 6g/kg compared to SGD 3g/kg group. In summary, SGD alleviates PIPN by reducing the expression and activity of TRPV1 and TLR4-MyD88 signaling. The application of SGD may be a promising therapeutic agent for pain control.

Discussion

Paclitaxel-induced peripheral neuropathy (PIPNe) is one of the major side effects faced in the treatment of tumor patients.³⁸ A traditional Chinese medicine concoction, SGD exerts a number of polypharmacological effects based on the ‘multi-component, multi-target and multi-pathways’ principle,⁴³ while exhibiting better clinical efficacy and negligible side effects compared to traditional analgesic medications.¹⁷ In an effort to further elucidate the analgesic mechanism of SGD, we employed a series of network pharmacology approaches to predict the mechanism of SGD in alleviation of PIPNe.

First, in the complex system of SGD, a total of 105 bioactive compounds and 40 potential protein targets of SGD on PIPNe were analyzed based on network pharmacology. Subsequently, SGD-PIPNe-potential target gene interaction and compound-target networks were constructed using 40 protein targets retrieved from three databases. As documented in the SGD-PIPNe interaction network, the 40 potential target genes were not independent, but exhibited linkage and interaction with each other. Additionally, we came across potential protein targets with high degree nodes occupying hub positions in the network. Subsequent analyses of the compound-target network illustrated that compounds with higher-degree were localized at the core sites. These targets and compounds exhibited a higher degree in this process, which suggested that they may confer an essential role in the meditation and treatment of PIPNe, and potentially account for the therapeutic effects of SGD treatment on PIPNe. Thereafter, 40 target genes were screened with the help of GO and KEGG enrichment analyses. Moreover, subsequent results from the GO and KEGG analyses indicated that the occurrence and development of PIPNe were directly associated with 386 biological functions and 20 signaling pathways, underscoring that the bioactive functions and signaling pathways of these genes may function as the potential mechanism of SGD in treating PIPNe.

The hard-done work of our peers has further shown that the up-regulation and sensitization of TRPV1 in peripheral sensory neurons serve as the mechanism of chronic pain, including the pain induced by paclitaxel.^{26,33} Herein, our network analyses revealed that TRPV1 occupied the core position in the PPI and compound-target network. Meanwhile, the results of KEGG analysis demonstrated that all the top three pathways, namely neuroactive ligand–receptor interaction, inflammatory mediator regulation of TRP channels, and calcium signaling pathway, were associated with TRPV1. In lieu of these findings, we speculated that SGD may exert its analgesic effect on PIPNe via regulation of the over-expression of TRPV1.

To ascertain our speculation, we further established paclitaxel-related PIPNe rat models and investigated the pain-relieving function of SGD on paclitaxel-related hypersensitivities. Subsequent experimentation revealed that SGD exerted a diminishing effect on thermal hyperalgesia in PIPNe models. Compared with the SGD 3g/kg dosage group, the high concentration SGD group (6 g/kg) achieved a better effect in relieving thermal hyperalgesia. Interestingly, the effect of SGD on alleviating mechanical hypersensitivities was not observed. This can be explained by the fact that TRPV1 is a detector of noxious heat.⁴⁴ Thus, it does not have high sensitivity to mechanical pain.⁴⁵ Moreover, repeat applications of paclitaxel and SGD are invasive stimulation, which may construct conditioned reflex regarding painful stimulation in rats.

Existing evidence indicates that TRPV1 is regulated by a number of mechanisms.^{46,47} To further investigate the underlying mechanism of TRPV1 regulation, we performed another KEGG pathway enrichment to analyze TRPV1 and the target genes of pain. We observed that TLRs signaling was enriched with a relatively high gene count and P value. Interestingly, toll-like receptors (TLRs) have been previously shown to be associated with neuropathic pain.^{48,49} Yan Li et al further illustrated that administration of paclitaxel can bring about an abrupt sensitization of TRPV1 via the TLR4-MyD88 signaling pathway.³³ Also, in accordance with our findings, the study carried out by Yuanyuan Li et al reported that EA alleviates PIPNe via mechanisms involving suppressing the over-expression of TLR4 signaling and TRPV1.⁵⁰ In light of the above-mentioned findings, we chose to further explore the significance of TLR4 and MyD88 expressions in regard to SGD’ alleviation of PIPNe.

Additional results of molecular biological validation demonstrated that TRPV1 and TLR4-MyD88 signaling were both highly expressed in DRGs of PIPN rats, whereas SGD administration brought about marked attenuation of the over-expression of TRPV1 and TLR4-MyD88 signaling, wherein high concentration of SGD (6g/kg) achieved a better effect compared with low concentration SGD (3g/kg). Moreover, recent findings have come to light illustrating the involvement of the up-regulated TLR4/PI3K/Akt/TRPV1 signalling pathway in paclitaxel-mediated adverse effects and PIPN in rodents.^{51,52} Meanwhile, existing evidence further suggests that peripheral neuropathy can be reversed with the use of a selective PI3K-signalling inhibitor.⁵¹ In addition, the study performed by Zhuangpeng Chang et al discovered that SGD administration could reduce the inflammation state by down-regulating TLR4/PI3K/Akt signalling, which is in line with our findings.⁵³ Taking these findings and data into consideration, it would be plausible to suggest that there are certain correlations between TLR4-PI3K signaling and TRPV1, and TLR4-PI3K signaling maybe the upstream of TRPV1.

Our study was the first-of-its-kind to explore the potential mechanism of SGD in treating PIPN and unveiled the mechanism wherein SGD exerts its analgesic effect by inhibiting the high expression of TRPV1 and TLR4-MyD88 signaling. However, how SGD regulates the TLR4/PI3K/Akt/TRPV1 signalling is still unclear, and warrants further exploration.

Conclusions

Collectively, our findings highlighted that SGD treatment relieves paclitaxel-induced chronic pain in rat models. Mechanistic data further indicated that SGD reverses the expressions of TRPV1 and TLR4-MyD88 signaling in DRGs of paclitaxel-treated rats, underscoring the use of SGD as a potential treatment for PIPN patients in clinics.

Disclosure

The authors report no conflicts of interest in this work.

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