Randomized Trial

Tanshinone IIA Exerts an Antinociceptive Effect in Rats with Cancer-induced Bone Pain

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Background: Cancer-induced bone pain (CIBP) is a common chronic pain characterized by 2 components, ongoing pain and breakthrough pain. Tanshinone IIA (TSN IIA) is a bioactive constituent of the traditional Chinese medicine Danshen, which has been reported to have an antinociceptive effect on neuropathic and inflammatory pain through downregulation of the late proinflammatory cytokine high-mobility group protein B1 (HMGB1).

Objective: To assess the antinociceptive effect of TSN IIA on CIBP.

Study Design: A randomized, double-blind, controlled animal trial was performed.

Setting: University lab in China.

Methods: A rat CIBP model was established by injecting Walker 256 mammary gland carcinoma cells into the intramedullary cavity of the tibia. Both ongoing pain, e.g., flinching and guarding, and breakthrough pain, e.g., limb use and von Frey threshold, were evaluated. The effects of intraperitoneally administered TSN IIA on pain behavior and the expression levels of spinal HMGB1, interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6 were determined. The effect of TSN IIA on the electrically evoked response of spinal wide-dynamic range (WDR) neurons was performed in vivo.

Results: TSN IIA dose-dependently inhibited cancer-induced ongoing pain and breakthrough pain. The expression levels of spinal HMGB1 and other inflammatory factors (IL-1β, TNF-α, and IL-6) were increased in the rat model, but they were suppressed by TSN IIA in a dose-dependent manner. Moreover, TSN IIA significantly inhibited the neuronal responses of WDR neurons in spinal deep layers.

Limitations: Further studies are warranted to ascertain how TSN IIA attenuates cancer-induced ongoing pain.

Conclusions: Our results indicate that TSN IIA attenuates cancer-induced ongoing pain and breakthrough pain, possibly via suppression of central sensitization in CIBP rats. Therefore, we have provided strong evidence supporting TSN IIA as a potential and effective therapy for relieving CIBP.

Key words: Cancer-induced bone pain, high-mobility group protein B1, Tanshinone IIA, ongoing pain, breakthrough pain

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Cancer pain is well known to result in chronic suffering, which greatly impacts life and health (1-3). Although new and innovative techniques have provided prolonged survival of patients with cancer pain, the manner in which there is improvement in the quality of life is still challenging (4). Among all types of cancer pain, cancer-induced bone pain (CIBP) is one of the most common. CIBP results primarily from metastasis of cancers to the bone, most commonly from lung, prostate, and breast carcinomas. Tumor metastasis involves bone remodeling and eventual bone fractures, which lead to incapacitating pain and immobility (5-8). It is estimated that over 33% of patients with an advanced cancer develop skeletal metastases and experience severe pain (9).

CIBP is characterized by ongoing and breakthrough pain. Ongoing pain is usually the first symptom of CIBP, which is persistent and deteriorates over time (10). With increasing bone destruction, breakthrough pain will occur. This type of bone pain is frequently considered as an incidental and spontaneous pain, indicative of touch- or movement-evoked and ambulatory pain, upon the presence of a triggering event (3). Of the 2 types of cancer pain, breakthrough pain is more intense and irregular, and it is more difficult to manage. It has been reported previously that breakthrough pain is present in approximately 75% of cases with CIBP, and it can severely affect the physical, psychological, and general quality of a patient's life (11). Currently, the most significant problem in the management of CIBP is that available chemotherapeutics are not adequate to control breakthrough pain, whereas they may be effective for ongoing pain.

Tanshinone IIA (TSN IIA) is one of the key bioactive phytochemicals of the Chinese medicinal herb Danshen (*Salvia miltiorrhiza*), which has been widely used in China for the treatment of cardiovascular disorders (12,13). Recently, it has been demonstrated that TSN IIA has significant antinociceptive effects on inflammatory and neuropathic pain and that its analgesic effect is at least partially mediated or modulated through inhibition of high-mobility group protein B1 (HMGB1) expression (14,15). HMGB1 is a nonhistone DNA-binding protein that is widely expressed in eukaryotic cells. HMGB1 is also a cytokine-like inflammatory mediator responsible for the regulation of gene transcription (16). Once released into the extracellular space, HMGB1 functions as a late inflammatory cytokine by binding to toll-like receptor 4 (TLR4), triggering an inflammatory response (17-19). HMGB1 has been demonstrated to be involved in arthritis (20), neuropathic pain (21), and diabetic pain (22). Besides, spinal HMGB1 has been linked to contribute to mechanical allodynia in a model of CIBP (23). Thus, inhibition of the release of spinal HMGB1 may exert an antinociceptive effect on CIBP. TSN IIA is a steroidal pigment (12,13) that has been shown to inhibit the upregulated expression of HMGB1 in cell cultures (24,25) and in focal ischemic rats (26). However, whether TSN IIA attenuates spinal HMGB1 release related to CIBP is unclear.

Therefore, in this study, we evaluated the capacity of TSN IIA to inhibit spinal HMGB1 release and its therapeutic potential in a rat model of CIBP. Our present investigation revealed that TSN IIA attenuates cancer-induced ongoing pain and breakthrough pain.

Methods

Animals

All surgical manipulations and behavioral tests of animals were approved by the Committee of Animal Use for Research and Education of the Inner Mongolia Medical University, Huhhot, Inner Mongolia, China. All efforts were made to minimize animal suffering and to reduce the number of animals used. Adult female Wistar rats, weighing 180 – 220 g, were from Inner Mongolia Medical University. The rats were housed 3 per cage with food and water available *ad libitum* under a 12 hour light-dark cycle (lights on at 06:00 AM).

Cell Preparation

Cells were prepared in accordance with previous studies (27). Briefly, 0.5 mL of Walker 256 rat mammary gland carcinoma cells (2×10^7 cells/mL) was injected into the abdominal cavity of the rats, and, 7 – 10 days later, ascitic fluid was extracted and centrifuged for 3 minutes at 1500 rpm. The collected cells were then diluted to 5×10^5 cells/10 μL of phosphate-buffered saline for injection. Cells used as a sham control were prepared at the same final concentration but boiled for 20 minutes.

Surgery

As described previously (27), rats were completely anesthetized by intraperitoneal (i.p.) injection of 50 mg/kg sodium pentobarbital, and the skin of the right leg was cut. A total of 5×10^5 carcinoma cells were slowly injected into the intramedullary cavity of the tibia. Upon removal of the syringe, the injection site was closed using bone wax, and penicillin was applied

to the wound. For the sham group, an equal volume of heat-treated carcinoma cells was administered instead.

Experimental Design

To evaluate the dose-dependent antinociceptive effects of TSN IIA on ambulatory pain, e.g., limb use, ongoing pain, e.g., flinching and guarding, and touch-evoked incident pain, e.g., von Frey filaments, of the CIBP rats, 24 rats were randomly divided into 4 groups (n = 6 each): CIBP + vehicle, CIBP + TSN IIA (10 mg/kg), CIBP + TSN IIA (20 mg/kg), and CIBP + TSN IIA (50 mg/kg). TSN IIA (Shanghai No. 1 Biochemical Pharmaceutical Company, Shanghai, China; batch number 130408) was injected i.p. on days 12 to 15, when nociceptive behavior reaches the highest level and is stable. Behavioral evaluation was performed one hour after the last TSN IIA injection on day 15. The same dose of saline was also administered to the vehicle-treated group.

To determine the time-dependent antinociceptive effects of TSN IIA in the CIBP rats, 24 rats were randomly divided into 4 groups (n = 6 each): sham + vehicle, sham + TSN IIA (20 mg/kg), CIBP + vehicle, and CIBP + TSN IIA (20 mg/kg). In the sham-operated group, saline instead of cancer cells was infused into the intramedullary cavity of the tibia. To exclude the potential influence of TSN IIA-induced motor dysfunction on behavioral evaluation, 24 healthy rats were randomly divided into 4 groups (n = 6 each): vehicle, TSN IIA (10 mg/kg), TSN IIA (20 mg/kg), and TSN IIA (50 mg/kg). A rotarod test was performed after the administration of vehicle or TSN IIA from days 1 to 4. To evaluate the effects of TSN IIA on spinal HMGB1 in the CIBP rats, HMGB1 protein expression was assayed one hour after TSN IIA injection on day 15. In addition, the expression levels of the pro-inflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 were determined by real-time reverse transcription polymerase chain reaction (RT-PCR) or an enzyme-linked immunosorbant assay (ELISA) one hour after TSN IIA injection on day 15 post cancer cell infusion.

Western Blot

Rats were sacrificed after anesthesia with an overdose of sodium pentobarbital (60 mg/kg, i.p.), and the L4-6 spinal dorsal horn was rapidly harvested. The selected region was homogenized in lysis buffer, and the crude homogenate was centrifuged at 4°C for 15 minutes at 1,000 g. The supernatants were collected, and the protein concentrations were measured. Equal amounts of protein samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by electrotransfer onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). After blockade with 3% nonfat dry milk in Tris-buffered saline containing 0.02% Tween-20 for one hour, the immunoblot was carried out by incubation at 4°C overnight with the primary antibody rabbit anti-HMGB1 (1:1000; BD Pharmigen, Piscataway, NJ, USA). Bound primary antibodies were detected with the anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:10,000; Amersham Pharmacia Biotech, Piscataway, NJ, USA). The reactions were enhanced with the chemiluminescence detection method (Amersham). The membranes were then re-probed with goat anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:1000; Santa-Cruz Biotechnology, Santa Cruz, CA, USA) and then HRP-conjugated secondary antibody (anti-goat 1:10,000; Amersham). The densities of HMGB1 and GAPDH immunoreactive bands were quantified with the software of Image J by investigators blind to the treatment groups. The same size of the rectangle was drawn around or near (for background) each band. The density of each band was quantified with the corresponding background subtraction. The ratios of HMGB1 to GAPDH were expressed as relative fold changes relative to the control.

RT-PCR

Total RNA was extracted with Trizol (Life Technologies, Grand Island, NY, USA). Single-stranded complementary DNA was synthesized with Superscript Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). The primers used are presented in Table 1. GAPDH served as an internal standard control. Real-time RT-PCR was performed using the SYBR Premix Ex Taq (Takara, Tokyo, Japan) on a sequence detection system (Applied Biosystems, Foster City, CA, USA). The amplification conditions were as follows: 3 minutes at 95°C, followed by 45 cycles of 10 seconds at 95°C for denaturation, and 45 seconds at 60°C for annealing and extension. The experiments were repeated twice on different days, and PCRs were run in triplicate for each experiment. The relative quantification of gene expression was estimated from the threshold amplification cycle number (Ct) using Sequence Detection System software (Applied Biosystems).

ELISA

TNF-α, IL-1β, and IL-6 in the spinal cord were analyzed using ELISA, according to the manufacturer's in-

Table 1. *Primers sequence for the rat genes characterized in this experiment.*

Genes	Primers		Accession number
TNF-α	Forward primer	5'-TGATCGGTCCCAACAAGG A-3'	AY427675
	Reverse primer	5'-TGCTTGGTGGTTTGCTACGA-3'	
IL-1β	Forward primer	5'-TGCTGATGTACCAGTTGGGG-3'	NM031512
	Reverse primer	5'-CTCCATGAGCTTTGTACAAG-3'	
IL-6	Forward primer	5'-GCCCTTCAGGAACAGCTATG-3'	NM012589
	Reverse primer	5'-CAGAATTGCCATTGCACAAC-3	
GAPDH	Forward primer	5'-CCCCCAATGTATCCGTTGTG-3'	NM01008
	Reverse primer	5'-TAGCCCAGGATGCCCTTTAGT-3'	

structions (BioSite, Paris, France). The cytokine concentrations were detected photometrically at 450 nm using a Fluoroskan Ascent cytofluorimeter (Thermo Electron, Milford, MA, USA), and the concentrations were calculated based on a standard curve generated by serial dilutions of purified recombinant mouse cytokines. All samples were analyzed in triplicate.

Behavioral Tests

Behaviorally, ongoing pain behaviors present with flinching and guarding, while breakthrough pain can be further divided into ambulatory pain, e.g., limb use, and touch- or movement-evoked pain, e.g., von Frey threshold pain. Rats were placed in a clear plastic observation box and allowed to acclimate for 30 minutes before performing the following tests: (1) limb use was evaluated by limping and guarding behavior scores during spontaneous ambulation. The ipsilateral hind limbs of the rats were rated on the following scale (28): 0, complete lack of limb use; 1, partial non-use; 2, limping and guarding; 3, limping; and 4, normal walking; (2) the numbers of spontaneous guards and flinches, representative of behaviors and indicative of ongoing pain, were recorded during a 2-minute observation period. Guarding was defined as the time that the hind paw was held aloft while not ambulatory. Flinching was defined as the number of times the animal held the hind paw aloft; (3) mechanical allodynia, which reflects behavior indicative of touch-evoked incident pain, was measured as reported previously (27). Briefly, the animals were habituated to the testing environment for 3 days (one hour per day) before testing, then placed into inverted plastic boxes (30 × 30 × 50 cm³) on an elevated mesh floor, and allowed to acclimate for 30 minutes before testing. The hind paws ipsilateral to the injection site were pressed with a logarithmic series of 8 calibrated Semmes-Weinstein monofilaments (von-Frey hairs; Stoelting, Kiel, WI, USA). Log stiffness of the hairs was determined by the log10 method (milligrams × 10). The filaments had the following log-stiffness values (the value in grams is shown in parentheses): 4.17 (1.479 g), 4.31 (2.041 g), 4.56 (3.630 g), 4.74 (5.495 g), 4.93 (8.511 g), 5.07 (11.749 g), 5.18 (15.136 g), and 5.46 (28.840 g). The range of monofilaments (1.479 – 28.840 g) produced a logarithmically graded slope that was used to interpolate the 50% response threshold of stimulus intensity, which was expressed as log10 (milligrams × 10). Each filament was applied 10 times, and the minimal value that caused more than 5 obvious withdrawals was recorded as the paw withdrawal threshold (PWT). The behavioral responses were used to calculate the 50% PWT by fitting a Gaussian integral psychometric function using a maximum-likelihood fitting method, as described previously (29). The researchers were blind to the groups for the behavioral testing.

Rotarod Test

Rotarod tests were performed in drug-administered but experiment-free rats. Briefly, after trained at the minimal speed for training sessions of 1 – 2 minutes at intervals of 30 – 60 minutes on the Ugo Basile 7650 Rotarod accelerator treadmill (Ugo Basile, Varese, Italy), the rats were placed onto the rotarod at a constant speed of 25 rpm. Then, the accelerator mode was selected on the treadmill, i.e., the rotation rate of the drum was increased linearly at 20 rpm. The time was recorded when the rat fell to the ground. The cut-off time was 30 seconds. Each rat was tested 30 minutes before drug administration as the control performance and then was tested once a day for 4 consecutive days during the drug administration. The time was expressed as the percentage of the animal's own mean control performance.

Electrophysiological Study

To examine whether TSN IIA induces central sensitization, in vivo electrophysiological activities were recorded on deep spinal wide-dynamic range (WDR) neurons (500 – 1200 μm) in the CIBP rats one hour after TSN IIA injection on postoperative day 15, the time point that cancer-induced pain usually reaches the highest level (27,30). Briefly, a glass micropipette filled with 2% Pontamine Sky Blue in 0.5 M sodium acetate (impedance = 10 – 15 MΩ at 1000 Hz) was inserted perpendicularly into the back of the L4-5 spinal cord segments and advanced dorsoventrally by a micromanipulator, while an electrical search stimulation (15 mA, 1 Hz, 1 ms pulse) was applied via 2 stainless steel needles inserted into the skin of the ipsilateral hind paw in order to identify the neurons that responded to the stimulation. According to previous studies, only WDR neurons in the spinal cord of CIBP rats show a significantly increased response to the stimuli (31); therefore, we focused on the WDR cells. Upon monitoring of a single unit, the WDR neurons were distinguished by their responses to both gentle brushing and a noxious pinch applied to the hind paw (31), and the threshold stimulus intensity for C-fiber response was detected (31). Then, 3 trains of stimuli (16 pulses with a 2-ms width, 0.5 Hz) were applied at a 10-minute interval, and the intensity was 3 times more potent than the C-fiber threshold intensity. The neuronal responses evoked by different types of fibers were separated by their latency: $A\beta$- (0 – 20 ms), $A\delta$- (20 – 90 ms), C-fiber response (90 – 300 ms), and post-discharge activity (300 – 800 ms) (31). Since C-response and post-discharge activities are considered to be nociceptively related (27,30,32), the C-response and post-discharge activities of the WDR neurons were collected for the evaluation of TSN IIA effects associated with the nociceptive stimuli.

Statistical Analysis

All data were expressed as the mean ± standard of error of the mean (SEM). A repeated measures analysis of variance (with Bonferroni confidence interval adjustment) was used for behavioral data. Data of western blot, RT-PCR, and ELISA analyses were analyzed using one-way analysis of variance. All statistical analyses were processed with SPSS software (version 16.0) (SPSS Inc., Chicago, IL, USA), and $P < 0.05$ was established as being statistically significant.

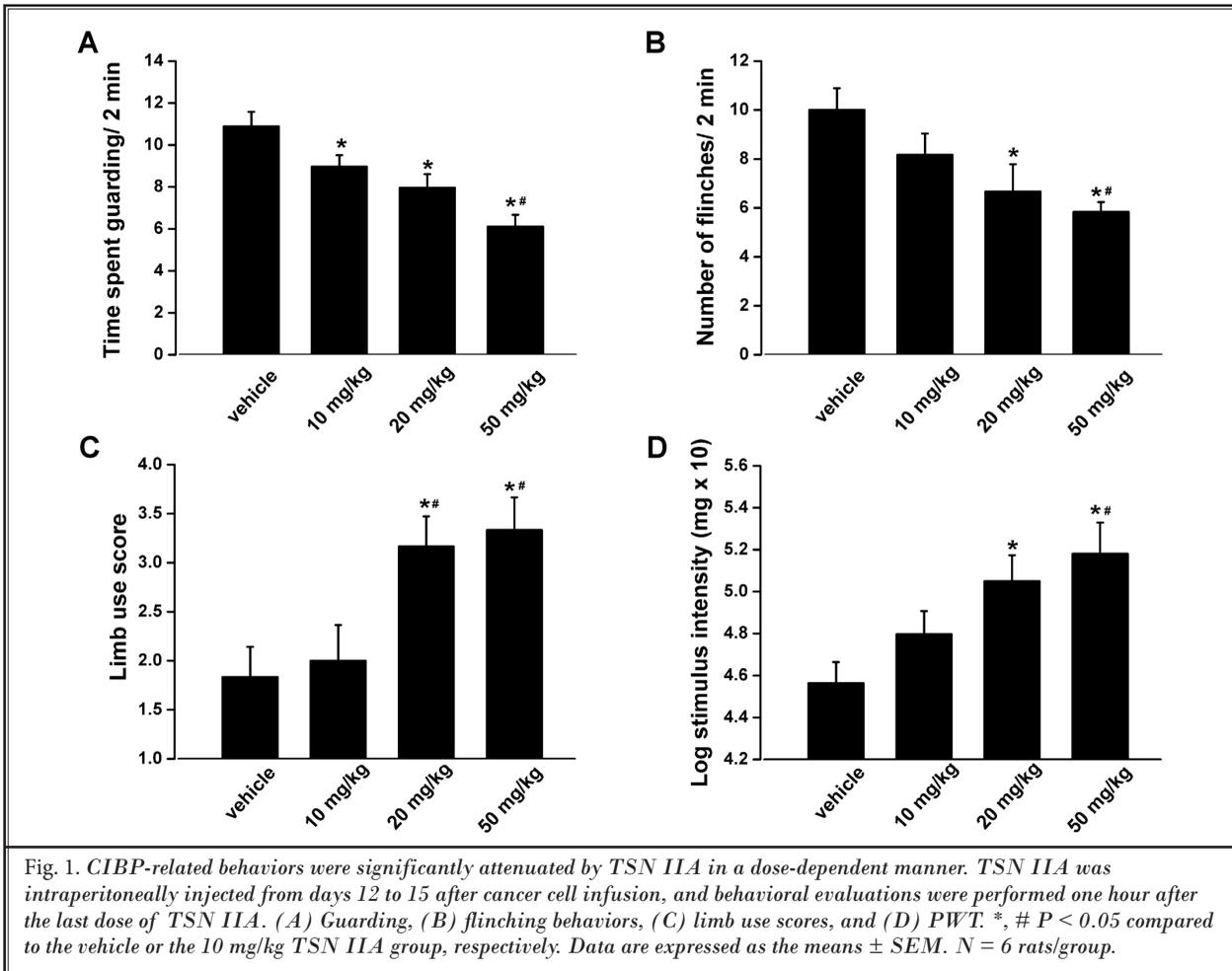## RESULTS

TSN IIA Reduces CIBP

Behavioral tests were analyzed for ongoing pain and breakthrough pain. We observed that the effects of TSN IIA on cancer pain-related behaviors occurred in a dose-dependent manner. TSN IIA at 10 mg/kg significantly reduced the time spent guarding. At a higher dose (20 and 50 mg/kg), TSN IIA administration reversed all tested cancer pain-related behaviors ($P < 0.05$) (Fig. 1). Moreover, the effects of TSN IIA at 50 mg/kg showed even greater inhibitory effects than those of TSN IIA at 10 mg/kg ($P < 0.05$) (Fig. 1).

Furthermore, compared with the sham-operated groups, the CIBP rats exhibited increased time spent guarding and number of flinches as well as reduced limb use and PWT to von Frey hairs ($P < 0.05$) (Fig. 2) from day 9. TSN IIA at 20 mg/kg administered from days 12 to 15 after cancer cell inoculation did not affect the pain-related behaviors in the sham group, compared to the vehicle-sham rats. However, it significantly reduced the ongoing flinching and guarding behaviors as well as increased the scores of limb use and the PWT on day 15 ($P < 0.05$) (Fig. 2).

It is known that the nociceptive behavioral results can be influenced by motor dysfunction (27). To exclude potential motor dysfunctions induced by TSN IIA, a rotarod performance test was performed in experiment-free rats. The results showed that repeated intrathecal injections (4 times, once per day) of TSN IIA did not affect the motor performance, compared with the saline injection group (Fig. 3), suggesting that the nociceptive effects of TSN IIA are not via the changed motor functions of the CIBP rats.

TSN IIA Inhibits CIBP-induced Spinal HMGB1 Expression

Spinal HMGB1 plays a modulating role in CIBP (23). In our study, the spinal HMGB1 expression levels were significantly increased from day 9 after cancer cell injection (data not shown), in accordance with previous reports (23). Therefore, we evaluated the effects of TSN IIA at 20 mg/kg on CIBP-induced spinal HMGB1 expression. Our results showed that the increased spinal HMGB1 expression in the CIBP rats was significantly suppressed by TSN IIA administration ($P < 0.05$) (Fig. 4). Notably, TSN IIA did not change the spinal HMGB1 expression in the sham-operated rats.

Fig. 1. *CIBP-related behaviors were significantly attenuated by TSN IIA in a dose-dependent manner. TSN IIA was intraperitoneally injected from days 12 to 15 after cancer cell infusion, and behavioral evaluations were performed one hour after the last dose of TSN IIA. (A) Guarding, (B) flinching behaviors, (C) limb use scores, and (D) PWT. *, # P < 0.05 compared to the vehicle or the 10 mg/kg TSN IIA group, respectively. Data are expressed as the means ± SEM. N = 6 rats/group.*

TSN IIA Inhibits CIBP-induced Spinal Cytokine Expression

Activation of the HMGB1–TRL4 pathway could potentially induce the upregulation of TNF-α, IL-1β, and IL-6. Therefore, the effects of TSN IIA on these cytokines were further investigated. TSN IIA was injected i.p. at 20 mg/kg from days 12 to 15 days after cancer cell injection, and cytokine expression levels were investigated on day 15. Consistent with the increased HMGB1 expression in the spinal dorsal cord of the CIBP rats, the mRNA and protein expression levels of TNF-α, IL-1β, and IL-6 were significantly higher than those of the sham-operated rats ($P < 0.05$) (Fig. 5). The injection of TSN IIA at 20 mg/kg, but not the vehicle, significantly reduced the spinal mRNA and protein expression of TNF-α, IL-1β, and IL-6 in the CIBP rats ($P < 0.05$) (Fig. 5).

TSN IIA Inhibits the CIBP-evoked Response of WDR Neurons

Additionally, our study demonstrated that the frequencies of the C-fiber and post-discharge responses of WDR neurons in the spinal dorsal horn were significantly enhanced in rats with CIBP ($P < 0.05$) (Fig. 6), in accordance with previous reports (27,30,31). TSN IIA treatment from days 12 to 15 significantly inhibited the electrically evoked C-fiber responses as well as the post-discharge responses ($P < 0.05$) (Fig. 6).

DISCUSSION

In the present investigation, it was demonstrated that clinically relevant concentrations of TSN IIA exerted a significant antinociceptive effect on CIBP, including both ongoing pain and breakthrough pain. Intraperito-
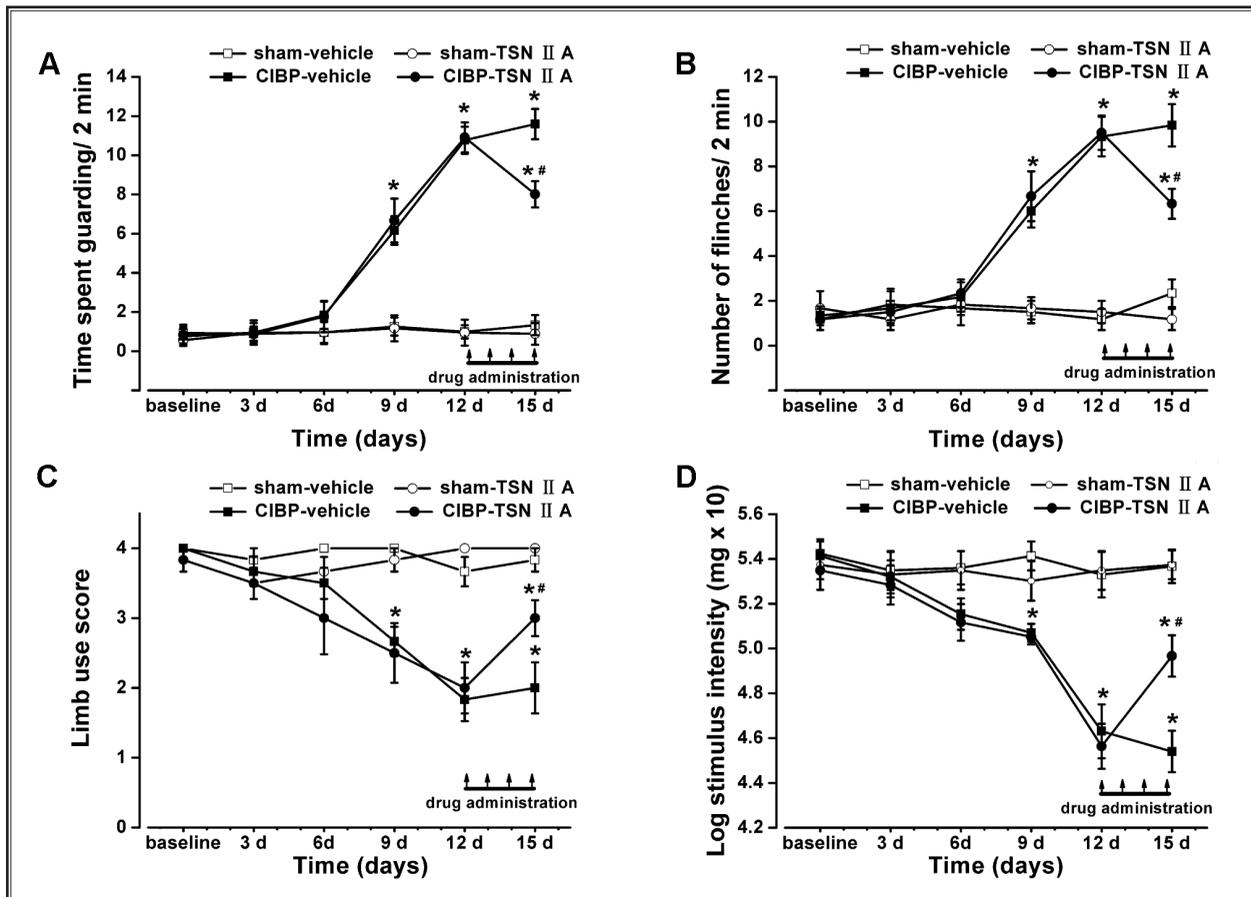
Fig. 2. *The effect of intraperitoneal administration of TSN IIA on pain-related behaviors in rats injected with cancer cells. TSN IIA (20 mg/kg) or saline was injected from days 12 to 15 after cancer cell implantation. (A) Time spent spontaneously guarding, (B) spontaneous flinches, (C) limb use, and (D) PWT to von Frey hairs (D). *, # P < 0.05 vs. the sham-vehicle or the CIBP-vehicle group, respectively. Data are expressed as the means ± SEM. N = 6 rats/group.*
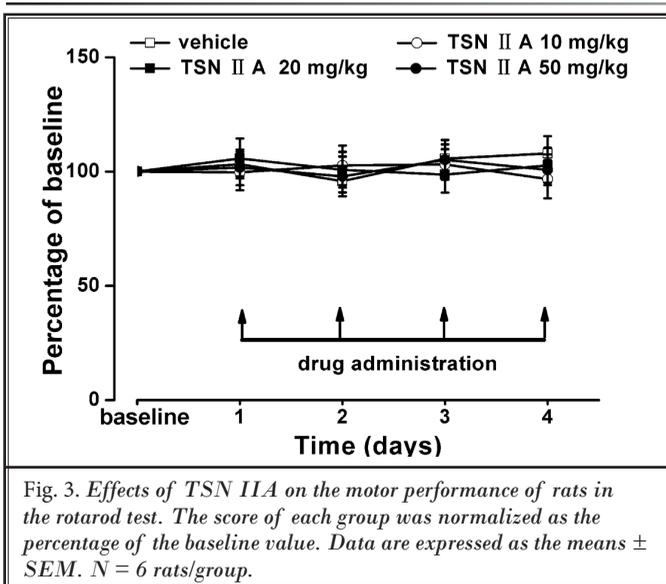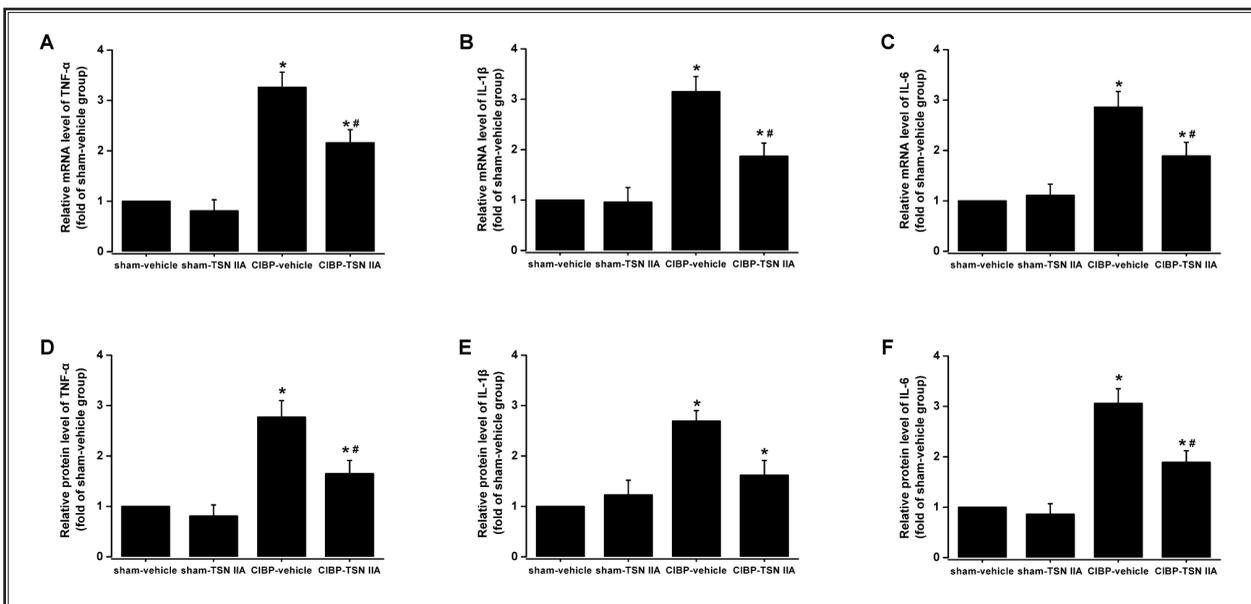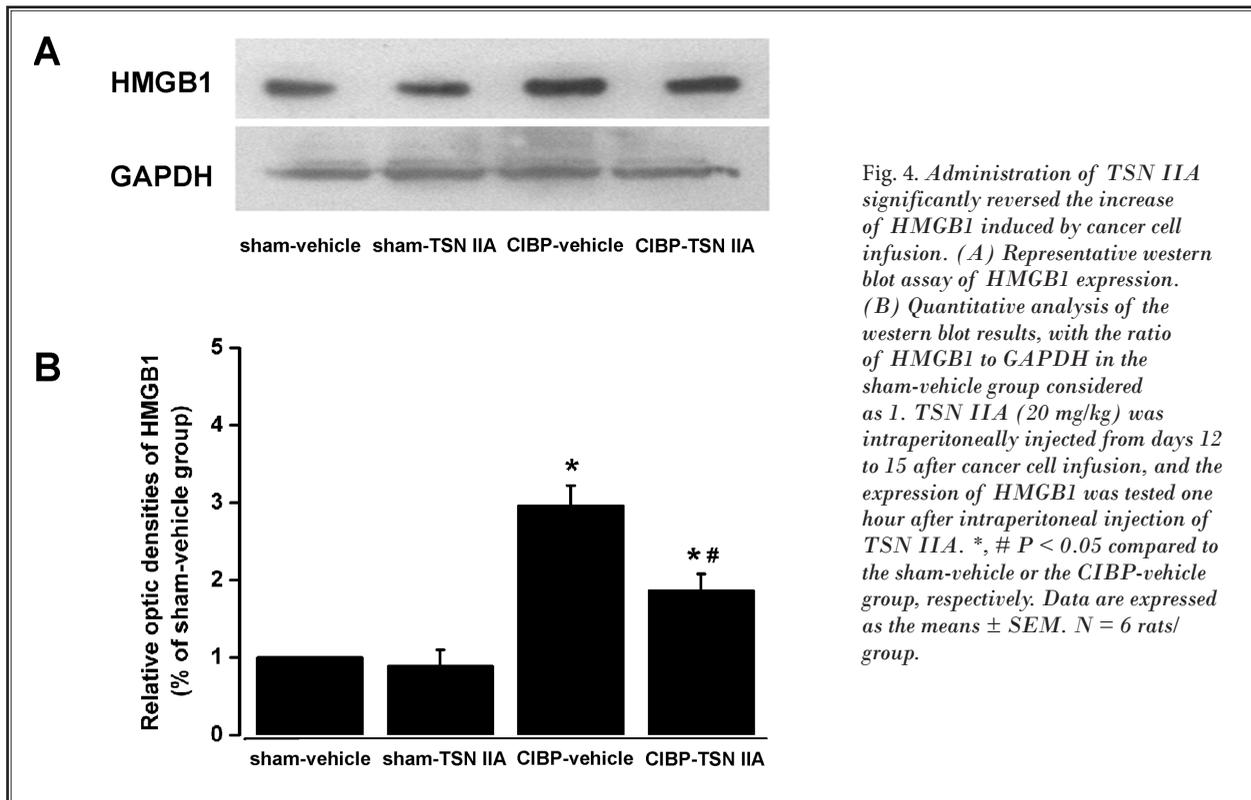

Fig. 3. *Effects of TSN IIA on the motor performance of rats in the rotarod test. The score of each group was normalized as the percentage of the baseline value. Data are expressed as the means ± SEM. N = 6 rats/group.*

neal administration of TSN IIA dose-dependently inhibited the release of spinal HMGB1 and its downstream proinflammatory cytokine production. Moreover, TSN IIA suppressed the firing frequency of C-fiber-evoked and post-discharge responses of WDR neurons. Together, these results indicate that TSN IIA has the potential for controlling CIBP by inhibiting HMGB1 and proinflammatory cytokines as well as suppressing excitable spinal WDR neurons.

Though CIBP is the most common symptom of cancer that metastasizes to bone, the mechanisms of CIBP initiation and maintenance are not well understood. CIBP elicits neurochemical changes unique from other chronic pain states, such as inflammatory and neuropathic pain (30). However, on the other hand, CIBP also shares some common
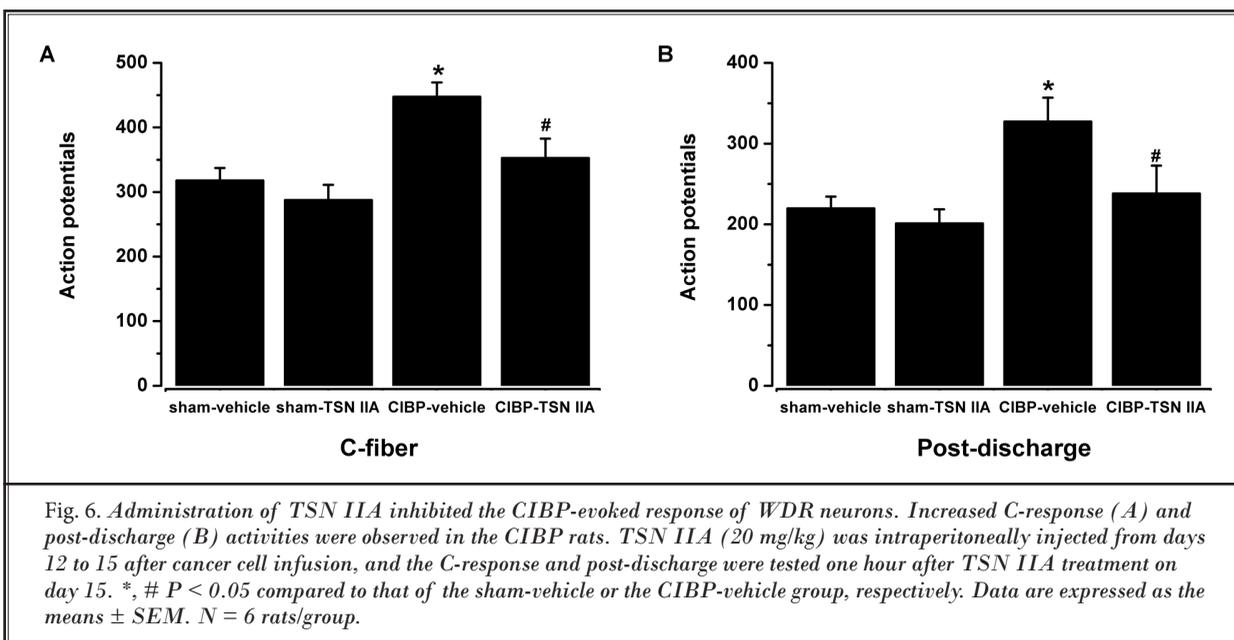
Fig. 4. *Administration of TSN IIA significantly reversed the increase of HMGB1 induced by cancer cell infusion. (A) Representative western blot assay of HMGB1 expression. (B) Quantitative analysis of the western blot results, with the ratio of HMGB1 to GAPDH in the sham-vehicle group considered as 1. TSN IIA (20 mg/kg) was intraperitoneally injected from days 12 to 15 after cancer cell infusion, and the expression of HMGB1 was tested one hour after intraperitoneal injection of TSN IIA. *, # P < 0.05 compared to the sham-vehicle or the CIBP-vehicle group, respectively. Data are expressed as the means ± SEM. N = 6 rats/ group.*

Fig. 5. *Administration of TSN IIA markedly inhibited the expression of cytokines analyzed by real-time PCR (A–C) and ELISA (D–F). TSN IIA (20 mg/kg) was intraperitoneally injected from days 12 to 15 after cancer cell infusion, and the expression of inflammatory cytokines was tested one hour after TSN IIA treatment on day 15. Changes of TNF-α (A, D), IL-1β (B, E), and IL-6 (C, F) in the spinal dorsal cord of the CIBP or sham rats at the mRNA and protein levels were tested. Data are expressed as fold changes of the sham-vehicle group. *, # P < 0.05 compared to that of the sham-vehicle or the CIBP-vehicle group, respectively. Data are expressed as the means ± SEM. N = 6 rats/group.*

Fig. 6. *Administration of TSN IIA inhibited the CIBP-evoked response of WDR neurons. Increased C-response (A) and post-discharge (B) activities were observed in the CIBP rats. TSN IIA (20 mg/kg) was intraperitoneally injected from days 12 to 15 after cancer cell infusion, and the C-response and post-discharge were tested one hour after TSN IIA treatment on day 15. *, # $P < 0.05$ compared to that of the sham-vehicle or the CIBP-vehicle group, respectively. Data are expressed as the means ± SEM. N = 6 rats/group.*

mechanisms with other chronic pain states. Therefore, drugs that are used to manage other chronic pain states, such as morphine and endomorphin-2, may also be effective for cancer pain (30). In recent studies, TSN IIA was observed to exert an antinociceptive effect on spinal nerve ligation-induced neuropathic pain (15) and complete Freund's adjuvant-induced inflammatory pain (14). However, whether TSN IIA can inhibit bone pain in CIBP rats is not clear.

Previously, HMGB1 has been demonstrated to play a role in regulating the production of inflammatory cytokines in intracerebral hemorrhage (33), spinal cord ischemic injury (34), and chronic pain (35). HMGB1 can be released by spinal astrocytes (36), which have been demonstrated to be activated in CIBP rats (37). Thus, HMGB1 may contribute to CIBP. In our study, the expression of HMGB1 was increased in the spinal cord of CIBP rats. Meanwhile, the expression and production levels of IL-1β, TNF-α, and IL-6 were upregulated. Furthermore, the increased expression of HMGB1 correlated with the development of pain behaviors in the CIBP rats. These results indicate that spinal HMGB1 is involved in the inflammatory process and the pain state of CIBP.

As a late proinflammatory cytokine, HMGB1 is characterized by a slow induction and a long duration, so inhibiting HMGB1 may produce a relatively widespread therapeutic effect. TSN IIA is an HMGB1 inhibitor. Previous studies have demonstrated that TSN IIA

inhibits HMGB1 release in cell cultures (24,38). Besides, in the chronic pain state, TSN IIA inhibits the expression of spinal HMGB1 in spinal nerve ligation-induced neuropathic pain rats (15) and in chronic pancreatitis-induced pain rats (35). In our study, TSN IIA reversed the release of spinal HMGB1 as well as the expression of IL-1β, TNF-α, and IL-6 in the CIBP rats. This mechanism might be explained by facilitation of the internalization of exogenous HMGB1 and enhancement of HMGB1 uptake by TSN IIA (24). Since TSN IIA has been demonstrated to have no inhibitory effect on most other cytokines (38,39), its inhibitory effects on the expression of proinflammatory cytokines might thus be related to its suppression of HMGB1 release in the CIBP rats.

The most common cancers, including lung, prostate, and breast carcinoma, commonly metastasize to bone. With the growth of tumors within bone, the local nociceptive system is sensitized such that neuroactive substances of peripheral sensory neurons are released. These pain signals are transmitted to the spinal cord and induce central sensitizations, mediating ambulatory pain and touch- or movement-evoked pain, which are the behavioral manifestations of breakthrough pain. It has been suggested that IL-1β, TNF-α, and IL-6 play an important role in the initiation and maintenance of central pain sensitization (40,41). Thus, the antinociceptive effect of TSN IIA on breakthrough pain might be related to the decreased production of proinflammatory cytokines. In addition, in the present

study, TSN IIA inhibited WDR neuronal hyperexcitability elicited by electrical stimulation of C-fibers, which are increased in the cancer pain state (27,30). WDR neurons are the major interconnecting neurons for nociceptive transmission, and the hyperexcitability of WDR neurons is related to central sensitization in chronic pain (42,43). Thus, in electrophysiology, the analgesic effect of TSN IIA on breakthrough pain is related to suppression of the central sensitization induced by hyperexcitable WDR neurons in CIBP rats. Our electrophysiological results are in accordance with the behavioral results, indicating that TSN IIA could effectively inhibit breakthrough pain.

In the present study, TSN IIA also reversed the ongoing pain behavior, e.g., flinching and guarding, in CIBP rats. The mechanisms underlying CIBP might be related to peripheral sensitization. In CIBP, osteoclasts are released to stimulate bone reabsorption and the release of proinflammatory factors into the tumor microenvironment, which sensitize primary afferent fibers (44). Within the tumor microenvironment, the acidosis induced by osteoclasts and mechanical damage to sensory fibers innervating the bone by tumor growth drive peripheral afferent activity (45,46). The sensitized primary afferent fibers lower thresholds, resulting in activation of nociceptive factors, enhancement of neuronal excitability, and release of inflammatory factors, which subsequently induce ongoing pain (45,47). Thus, intraperitoneal administration of TSN IIA could exert antinociceptive effects on cancer-induced ongoing pain behaviors by inhibiting peripheral proinflammatory cytokines and suppressing sensitization and activation of primary afferent fibers in the tumor microenvironment.

Greater understanding of the mechanisms of TSN IIA mediating or modulating ongoing pain in CIBP rats will require future investigation.

TSN IIA has been used clinically, and it exerts an anticancer activity (48). However, it shows poor bioavailability with conventional delivery formulations through oral administration. The high hydrophobicity and poor membrane permeability of TSN IIA might be responsible for impeding its absorption when given orally. Therefore, structural modifications are needed to improve the solubility problem and increase the absorption of TSN IIA. So, additional clinical studies are warranted to better demonstrate the analgesic efficacy and tolerability of TSN IIA derivatives in humans.

Conclusions

The results of the present investigation demonstrated that TSN IIA could significantly attenuate cancer-induced ongoing pain and breakthrough pain by suppressing the central sensitization in CIBP rats. Furthermore, this investigation provides strong evidence supporting TSN IIA as a potential and effective therapy for relieving cancer-induced pain.

Competing Interest Statement

Dr. Wei Hao, Lei Chen, and Li-fang Wu contributed equally to this work. There was no financial relationship with any organization that might have an interest in the submitted work during the previous 3 years, and there are no other relationships or activities that could appear to have influenced the submitted work. Alan David Kaye, and Shi-yuan Xu are equal contributors of this work.

References

1. Foley KM. Controlling cancer pain. *Hosp Pract (Minneap)* 2000; 35:101-108, 111-112.

2. Foley KM. Advances in cancer pain. *Arch Neurol* 1999; 56:413-417.

3. Portenoy RK, Lesage P. Management of cancer pain. *Lancet* 1999; 353: 1695-1700.

4. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005; 55:74-108.

5. Banning A, Sjogren P, Henriksen H. Treatment outcome in a multidisciplinary cancer pain clinic. *Pain* 1991; 47:129-134; discussion 127-128.

6. Mercadante S. Malignant bone pain: pathophysiology and treatment. *Pain* 1997; 69:1-18.

7. Reale C, Turkiewicz AM, Reale CA. Antalgic treatment of pain associated with bone metastases. *Crit Rev Oncol Hematol* 2001; 37:1-11.

8. Mundy GR. Mechanisms of bone metastasis. *Cancer* 1997; 80:1546-1556.

9. Thurlimann B, de Stoutz ND. Causes and treatment of bone pain of malignant origin. *Drugs* 1996; 51:383-398.

10. Kerba M, Wu JS, Duan Q, Hagen NA, Bennett MI. Neuropathic pain features in patients with bone metastases referred for palliative radiotherapy. *J Clin Oncol* 2010; 28:4892-4897.

11. Laird BJ, Walley J, Murray GD, Clausen E, Colvin LA, Fallon MT. Characterization of cancer-induced bone pain: An exploratory study. *Support Care Cancer* 2011; 19:1393-1401.

12. Ji XY, Tan BK, Zhu YZ. Salvia miltiorrhiza and ischemic diseases. *Acta Pharmacol Sin* 2000; 21:1089-1094.

13. Wu TW, Zeng LH, Fung KP, Wu J, Pang H, Grey AA, Weisel RD, Wang JY. Effect of sodium tanshinone IIA sulfonate in the rabbit myocardium and on human cardiomyocytes and vascular endothelial cells. *Biochem Pharmacol* 1993; 46:2327-2332.

14. Sun S, Yin Y, Yin X, Cao F, Luo D, Zhang

T, Li Y, Ni L. Anti-nociceptive effects of Tanshinone IIA (TIIA) in a rat model of complete Freund's adjuvant (CFA)-induced inflammatory pain. *Brain Res Bull* 2012; 88:581-588.

15. Ma YQ, Chen YR, Leng YF, Wu ZW. Tanshinone IIA Downregulates HMGB1 and TLR4 expression in a spinal nerve ligation model of neuropathic pain. *Evid Based Complement Alternat Med* 2014; 2014:639563.

16. Lotze MT, Tracey KJ. High-mobility group box 1 protein (HMGB1): Nuclear weapon in the immune arsenal. *Nat Rev Immunol* 2005; 5:331-342.

17. Czura CJ, Wang H, Tracey KJ. Dual roles for HMGB1: DNA binding and cytokine. *J Endotoxin Res* 2001; 7:315-321.

18. Andersson U, Erlandsson-Harris H, Yang H, Tracey KJ. HMGB1 as a DNA-binding cytokine. *J Leukoc Biol* 2002; 72:1084-1091.

19. Yamada S, Maruyama I. HMGB1, a novel inflammatory cytokine. *Clin Chim Acta* 2007; 375:36-42.

20. Agalave NM, Larsson M, Abdelmoaty S, Su J, Baharpoor A, Lundback P, Palmblad K, Andersson U, Harris H, Svensson CI. Spinal HMGB1 induces TLR4-mediated long-lasting hypersensitivity and glial activation and regulates pain-like behavior in experimental arthritis. *Pain* 2014; 155:1802-1813.

21. Nakamura Y, Morioka N, Abe H, Zhang FF, Hisaoka-Nakashima K, Liu K, Nishibori M, Nakata Y. Neuropathic pain in rats with a partial sciatic nerve ligation is alleviated by intravenous injection of monoclonal antibody to high mobility group box-1. *PLOS ONE* 2013; 8:e73640.

22. Ren PC, Zhang Y, Zhang XD, An LJ, Lv HG, He J, Gao CJ, Sun XD. High-mobility group box 1 contributes to mechanical allodynia and spinal astrocytic activation in a mouse model of type 2 diabetes. *Brain Res Bull* 2012; 88:332-337.

23. Tong W, Wang W, Huang J, Ren N, Wu SX, Li YQ. Spinal high-mobility group box 1 contributes to mechanical allodynia in a rat model of bone cancer pain. *Biochem Biophys Res Commun* 2010; 395:572-576.

24. Zhang Y, Li W, Zhu S, Jundoria A, Li J, Yang H, Fan S, Wang P, Tracey KJ, Sama AE, Wang H. Tanshinone IIA sodium sulfonate facilitates endocytic HMGB1 uptake. *Biochem Pharmacol* 2012; 84:1492-1500.

25. Jang SI, Jeong SI, Kim KJ, Kim HJ, Yu HH, Park R, Kim HM, You YO. Tanshi-

none IIA from Salvia miltiorrhiza inhibits inducible nitric oxide synthase expression and production of TNF-alpha, IL-1beta and IL-6 in activated RAW 264.7 cells. *Planta Med* 2003; 69:1057-1059.

26. Wang L, Zhang X, Liu L, Cui L, Yang R, Li M, Du W. Tanshinone II A down-regulates HMGB1, RAGE, TLR4, NF-kappaB expression, ameliorates BBB permeability and endothelial cell function, and protects rat brains against focal ischemia. *Brain Res* 2010; 1321:143-151.

27. Chen L, Zhu L, Wang K, Wang W, Mei XP, Liu T, Zhang FX, Wang W, Chen T, Li YQ. Antinociceptive effect of prostatic acid phosphatase in a rat model of cancer-induced bone pain. *Pain Physician* 2013; 16:533-546.

28. Gui Q, Xu C, Zhuang L, Xia S, Chen Y, Peng P, Yu S. A new rat model of bone cancer pain produced by rat breast cancer cells implantation of the shaft of femur at the third trochanter level. *Cancer Biol Ther* 2013; 14:193-199.

29. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods* 1994; 53:55-63.

30. Chen L, Wang K, Yang T, Wang W, Mei X, Zhu C, Wang W, Zhang F, Li Y. Downregulation of spinal endomorphin-2 correlates with mechanical allodynia in a rat model of tibia cancer. *Neuroscience* 2014; 286C:151-161.

31. Urch CE, Donovan-Rodriguez T, Dickenson AH. Alterations in dorsal horn neurones in a rat model of cancer-induced bone pain. *Pain* 2003; 106:347-356.

32. Lu N, Han M, Yang ZL, Wang YQ, Wu GC, Zhang YQ. Nociceptin/Orphanin FQ in PAG modulates the release of amino acids, serotonin and norepinephrine in the rostral ventromedial medulla and spinal cord in rats. *Pain* 2010; 148:414-425.

33. Lei C, Lin S, Zhang C, Tao W, Dong W, Hao Z, Liu M, Wu B. High-mobility group box1 protein promotes neuroinflammation after intracerebral hemorrhage in rats. *Neuroscience* 2013; 228:190-199.

34. Wang Q, Ding Q, Zhou Y, Gou X, Hou L, Chen S, Zhu Z, Xiong L. Ethyl pyruvate attenuates spinal cord ischemic injury with a wide therapeutic window through inhibiting high-mobility group box 1 release in rabbits. *Anesthesiology* 2009; 110:1279-1286.

35. Wang YS, Li YY, Wang LH, Kang Y, Zhang J, Liu ZQ, Wang K, Kaye AD, Chen

L. Tanshinone IIA attenuates chronic pancreatitis-induced pain in rats via downregulation of HMGB1 and TRL4 expression in the spinal cord. *Pain Physician* 2015; 18:E615-628.

36. O'Connor KA, Hansen MK, Rachal PC, Deak MM, Biedenkapp JC, Milligan ED, Johnson JD, Wang H, Maier SF, Tracey KJ, Watkins LR. Further characterization of high mobility group box 1 (HMGB1) as a proinflammatory cytokine: Central nervous system effects. *Cytokine* 2003; 24:254-265.

37. Schwei MJ, Honore P, Rogers SD, Salak-Johnson JL, Finke MP, Ramnaraine ML, Clohisy DR, Mantyh PW. Neurochemical and cellular reorganization of the spinal cord in a murine model of bone cancer pain. *J Neurosci* 1999; 19:10886-10897.

38. Li W, Li J, Ashok M, Wu R, Chen D, Yang L, Yang H, Tracey KJ, Wang P, Sama AE, Wang H. A cardiovascular drug rescues mice from lethal sepsis by selectively attenuating a late-acting proinflammatory mediator, high mobility group box 1. *J Immunol* 2007; 178:3856-3864.

39. Zhu S, Li W, Li J, Sama AE, Wang H. Caging a beast in the inflammation arena: Use of Chinese medicinal herbs to inhibit a late mediator of lethal sepsis, HMGB1. *Int J Clin Exp Med* 2008; 1:64-75.

40. Sun T, Luo J, Jia M, Li H, Li K, Fu Z. Small interfering RNA-mediated knockdown of NF-kappaBp65 attenuates neuropathic pain following peripheral nerve injury in rats. *Eur J Pharmacol* 2012; 682:79-85.

41. Latremoliere A, Woolf CJ. Central sensitization: A generator of pain hypersensitivity by central neuralplasticity. *J Pain* 2009; 10:895-926.

42. Li KC, Chen J. Altered pain-related behaviors and spinal neuronal responses produced by s.c. injection of melittin in rats. *Neuroscience* 2004; 126:753-762.

43. Khasabov SG, Hamamoto DT, Harding-Rose C, Simone DA. Tumor-evoked hyperalgesia and sensitization of nociceptive dorsal horn neurons in a murine model of cancer pain. *Brain Res* 2007; 1180:7-19.

44. Lozano-Ondoua AN, Symons-Liguori AM, Vanderah TW. Cancer-induced bone pain: Mechanisms and models. *Neurosci Lett* 2013; 557:52-59.

45. Mantyh PW. A mechanism based understanding of cancer pain. *Pain* 2002; 96:1-2.

46. Mantyh PW, Clohisy DR, Koltzenburg M, Hunt SP. Molecular mechanisms

of cancer pain. *Nat Rev Cancer* 2002; 2:201-209.

47. Jakobsson PJ. Pain: How macrophages mediate inflammatory pain via ATP signaling. *Nat Rev Rheumatol* 2010; 6:679-681.

48. Zhou L, Zuo Z, Chow MS. Danshen: An overview of its chemistry, pharmacology, pharmacokinetics, and clinical use. *J Clin Pharmacol* 2005; 45:1345-1359.