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Topical treatment with *Tong-Luo-San-Jie* Gel alleviates bone cancer pain in rats

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Abstract

Ethnopharmacological relevance—The herbal analgesic gel *Tong-Luo-San-Jie* (TLSJ) and its modifications are used in traditional Chinese medicine to manage cancer pain. However, its mechanisms are still unknown.

Aim of the study—To investigate the effects and mechanisms of TLSJ gel on bone cancer pain in a rat model.

Materials and Methods—A bone cancer pain rat model was established by inoculating Walker 256 rat carcinoma cells directly into the right tibial medullary cavity of Sprague-Dawley rats (150–170 g); Phosphate buffered saline (PBS) tibial inoculation was used as control. Cancer-bearing rats were treated twice a day with external TLSJ gel (0.5 g/cm²/day) or inert gel control for 21 days ($n=10$ /group). Behavioral tests such as mechanical threshold and paw withdrawal latency (PWL) were carried out. Osteoclastic activities were determined and carboxyterminal pyridinoline cross-linked type I collagen telopeptides (ICTP) and bone-specific alkaline phosphatase (BAP) concentrations were detected with ELISA after treatment. Adverse effects were monitored, and biochemical and histological tests were performed in naïve rats treated with local TLSJ gel for six weeks.

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#They made equal contributions to this work.

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Results—TLSJ treatment significantly restored bone cancer-induced decrease of PWL and mechanical threshold compared to inert gel. It also decreased the level of blood serum ICTP and BAP and inhibited osteoclast activities. No adverse effects or abnormal biochemical and histological changes were detected after TLSJ treatment.

Conclusion—The present study shows that TLSJ significantly inhibits bone cancer-induced thermal and mechanical sensitization. It suggests that the gel may be useful in managing cancer pain and that it may act by inhibiting osteoclastic activity.

Keywords

Chinese herbs; bone cancer pain; *Tong-Luo-San-Jie (TLSJ)* gel; type I collagen carboxy- terminal telopeptide; bone-specific alkaline phosphatase; osteoclast

1. Introduction

Cancer pain, the most common symptom of cancer, can result both from the direct effects of malignancy and from the treatment. In 56–79% of cases, the pain is tumor related; in 5–62% of cases it is related to the treatment (Banning et al., 1991; Gutgsell et al., 2003). A recent systematic review reports that pain was documented in 64% of patients with metastatic, advanced, or terminal disease, in 59% of those on anticancer treatment, and in 33% of individuals who had been cured of cancer (van den Beuken-van Everdingen et al., 2007).

Opioids are the main pharmacological therapy for managing cancer pain (Pargen and Hailey, 1999; Radha Krishna et al., 2010; Virik and Glare, 2000). However, their adverse effects, including nausea, constipation, urinary retention, dizziness, and dysphoria, markedly limit their use (Bowdle, 1998; Cherny et al., 2001; Inturrisi, 1989; Pasternak, 1988). Non-steroidal anti-inflammatory drugs are used to manage painful metastases but also have severe side effects such as gastrointestinal disturbances (Schaffer et al., 2006; Scheiman, 2001) and cardiovascular risks (Davies and Jamali, 2004).

In recent years, Chinese herbal medicine has played an important role in the treatment of cancer pain. It is reported that 41–62% of cancer patients use herbs as a complementary or alternative medical therapy (Gozum et al., 2003; Richardson et al., 2000). Because oral ingestion of herbal medicine may cause nausea, vomiting, or diarrhea, external treatment might be an acceptable alternative route for treating localized, cancer-related pain such as bone pain. Clinical observations showed that Chinese herbal medicine may be useful for managing cancer pain in patients (Chen et al., 2006; Xu et al., 2007). In a previous study, we demonstrated that an herbal analgesic gel, Xiaotan Tongluo (XTTL), prepared from four herbs, Tiannanxing (*Arisaema heterophyllum* Blume, rhizome), Banxia (*Pinellia ternate* (Thunb) Makino, tuber), Shancigu (*Cremastra appendiculata* (D. Don) Makino, pseudobulb) and Weilingxian (*Clematis chinensis* Osbeck, root), significantly alleviated the mechanical pain in a bone cancer pain animal model (Yu et al., 2009). However, the mechanisms of action of topically used herbs are largely unknown.

Osteoclastic activity is involved in bone metastasis-induced cancer pain, and osteolytic cancers produce significant proliferation of osteoclasts (Adami, 1997; Clohisy et al., 2000). Recent experiments in a bone cancer pain model show that osteoclasts have an essential role in cancer-induced bone loss and contribute to the etiology of bone cancer pain (Honore et al., 2000; Luger et al., 2001). Carboxyterminal pyridinoline cross-linked type I collagen telopeptides (ICTP) and bone alkaline phosphatase (BAP) are respectively the best characterized markers of osteoclastic (bone resorption) and osteoblastic (bone formation) activity (Smith, 2006). ICTP and BAP are significantly elevated in cancer patients (Koizumi et al., 1995; Tamada et al., 2001), which, when used in combination, may be reliable serum

markers for detecting bone metastases. In our previous study, measurements on day 21 post-cancer cell inoculation demonstrated that herbal analgesic gel decreased serum ICTP and BAP (Yu et al., 2009).

We recently developed another herbal analgesic gel, *Tong-Luo-San-Jie* (TLSJ), based on a classical formula for pain management, *San Qi He Bi Zhen*. Our TLSJ formula is prepared from seven herbs, different from those used in the XTTL gel. In traditional Chinese medicine (TCM), TLSJ is used to activate Qi and Blood circulation and to warm the channels to alleviate pain (Li et al., 2003; Sheu et al., 2009; Su et al., 2012; Yuan et al., 2004). According to TCM theory, qi is defined as the vital energy necessary for growth, development, and the maintenance of healthy physiological functions in the human body. In addition, qi promotes the formation of blood and drives its circulation (Lao, 1999).

The aim of this study was to evaluate the effects and safety of TLSJ gel and to explore the mechanisms of its action on cancer pain in a bone cancer pain rat model by assessing both mechanical and thermal sensitivity, the time course of serum ICTP and BAP, and the activity of osteoclasts and osteoblasts in the tibia. We hypothesized that our TLSJ gel alleviates bone cancer-induced pain by inhibiting osteoclastic activity.

2. Materials and Methods

2.1. Herbal preparation and extraction

TLSJ gel is composed of extracts prepared from seven herbs: *Haifengteng* (*Piper kadsura* (Choisy) Ohwi, stem) 30 g, *Luoshiteng* (*Trachelospermum jasminoides* (Lindl.) Lem., stem) 30 g, *Chuanshanlong* (*Dioscorea nipponica* Makino, rhizome) 50 g, *Yanhusuo* (*Corydalis yanhuso* W.T. Wang, rhizome) 30 g, *Chuanlianzi* (*Melia toosendan* Sieb. et Zucc, fruit) 10 g, *Ruxiang* (*Boswellia carterii* Birdw., defatted gum resin) 10 g, and *Moyao* (*Commiphora myrrha* Engl, resin) 10 g. The herbs, processed with traditional methods after harvest (Bensky and Gamble, 1993), were purchased from the Huayu Pharmaceutical Company (Shanghai, China), which has retained a complete set of samples of the authenticated herbs. Each extract was monitored for contaminants (heavy metals, pesticides, and mycotoxins) prior to formulation. The herbs were pulverized, admixed in the proper ratio, and extracted twice with 70% ethanol. The extract was subjected to decompression ethanol recovery (50°C, 0.08 MPa). Carbopol® 940 was used as the binding agent in the preparation of the gel, the formulation of which consists of Carbopol® 940, 0.8%; distilled water, 84.2%; herbal extract, 7.0%; propylene glycol, 4.0%; glycerol, 2.0%; and azone 2.0%. The pH of the gel was 6.5. Each g of gel contains 5 g equivalent of raw herbs (Liu et al., 2009; Sareen et al., 2011).

The chemical profile of the formulated gel was established by ultra performance liquid chromatography (UPLC) analysis (He et al., 2011; Wang et al., 2011) employing gradient elution with acetonitrile and 0.1% formic acid as mobile phases A and B, respectively. The ESI source was operated in the positive ionization mode. Data were collected between minutes 1 and 50; the mass-scan range was 100–800. Characteristic UPLC peaks (profile) for quality control/quality assurance are presented in Figure 1.

2.2. Animals

Female Sprague-Dawley rats weighing 150–170 g were obtained from the Shanghai Slac Laboratory Animal Co., Ltd. The animals were kept under controlled environmental conditions (24°C ± 0.5°C relative humidity 50–60%, 7am to 7pm alternate light-dark cycles, food and water *ad libitum*). The cage floors were covered with wood shavings to minimize the possibility of painful contact with a hard surface. The animal protocol was approved by the institutional animal care and use committees of the Shanghai University of Traditional

Chinese Medicine (China) and the University of Maryland School of Medicine (USA). The research was conducted in accordance to the “Guide for the Care and Use of Laboratory Animals” (National Research Council, 2010).

2.3. Experimental design

Four sets of experiments were carried out for: adverse effects, pain assessment, serum levels of ICTP and BAP, and osteoclastic and osteoblastic activity.

In experiment 1, 30 normal rats were used to assess TLSJ toxicity/adverse effects for six weeks. Behavioral observation, blood biochemistry tests, and histopathological examinations were performed.

In experiment 2, rats weighing 150–170 g were randomly divided into three groups ($n=10$ per group): (1) sham: vehicle (PBS) inoculation plus topical administration of inert gel; (2) vehicle: Walker 256 cell inoculation plus topical administration of inert gel; (3) treatment: Walker 256 cell inoculation plus topical administration of TLSJ gel at a dosage of 0.5 g/cm²/day, which effectively alleviated thermally evoked pain in our pilot study. To investigate the effects of the gel on cancer-induced pain, TLSJ gel (treatment group) or control inert gel (vehicle and sham groups) were evenly applied on the skin of tumor-bearing tibias twice a day at 8:00 AM and 20:00 PM for a total of 21 days beginning one day after Walker 256 cell implantation.

In experiment 3 (determination of serum levels of ICTP and BAP), rats were divided into sham cancer, cancer + vehicle, and cancer + TLSJ groups. The three groups were subdivided into 5 groups each for time points at baseline, days 5, 11, 17, and 21 ($n=5$ per group). At each time point, trunk blood samples (1.5 ml) were collected for ICTP and BAP measurements.

In experiment 4, two sets of three groups of rats were euthanized at baseline and at day 21 post-cancer cell implantation. The tumor-bearing tibias were removed to identify osteoclasts and osteoblasts.

2.4. Toxicity/adverse effects assessment

2.4.1. Observation of unusual behavioral changes—Normal rats ($n=30$, half male and half female) were randomly divided into three groups ($n=10$ per group) and respectively treated twice a day for six weeks with 1) low dosage TLSJ at 0.5 g/cm²/day, 2) high dosage at 1 g/cm²/day, or 3) inert gel control. In order to ascertain that the TLSJ is a safe formula, we also administered a higher dosage than that used in the behavioral tests. The rats were weighed twice a week. Adverse effects were closely monitored daily in the manner we reported previously (Fan et al., 2005), and in compliance with Chinese (China State Administration of Traditional Chinese Medicine, 1999) and American (FDA, 2004) guidelines for developing new Chinese herbal medicines and botanical drugs. Standard pharmacological categories of toxic and adverse behavioral reactions were applied following Dr. Gad's methods (Gad, 2002).

2.4.2. Blood biochemistry tests—Heart blood was collected at the end of six weeks and centrifuged (1310 g) for 15 min at 4°C. The plasma was collected and stored at −80°C until biochemistry tests were conducted. Sera creatine kinase (CK), CK-MB (M, muscle subunit; B, Brain type subunit), lactate dehydrogenase (LDH), hydroxybutyrate dehydrogenase (HBDH), total protein (TP), albumin (ALB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (TBIL), creatinine (CREA), blood

urea nitrogen (BUN), uric acid (UA), and cystatin C (CysC) were tested using commercial kits (Roche, Switzerland) to detect any TLSJ effects on heart, liver, or kidney function.

2.4.3. Gross necropsy and histopathology—Each animal was subjected to full examination of the external surface of the body, all orifices, and the thoracic, abdominal and cranial cavities and their organs after the end of treatment on day 42. No rats died during the experiment.

The heart, liver, kidney, spleen, and local skin were rinsed, then fixed in 10% neutral buffered formalin, embedded in paraffin, cut into 5 μ m cross-sections using a rotary microtome, (Leica RM 2165) and stained with hematoxylin and eosin (HE). Histopathological observation was conducted under a light microscope (Leica DM 2500). All observations were performed by investigators blinded to treatment group assignments.

2.5. Induction of bone cancer

2.5.1. Preparation of cells—Walker 256 cells, rat mammary gland carcinoma cells capable of inducing bone metastases, were obtained from the Shanghai Sino-British SIPPR/B&K Lab Animal Ltd. The cells (1×10^7 cells/ml) were injected into the abdominal cavities of the rats (70–90 g), and the ascitic fluid was extracted after 6–7 days. The cells were collected, washed with PBS, resuspended in a concentration of $3.5 \times 10^5/6 \mu$ l PBS, and kept on ice until being injected into rats.

2.5.2. Implantation of cancer cells—The rats (150–170 g) were anesthetized with pentobarbital (50 mg/kg) administered intraperitoneally (i.p.) after the right knee joint was shaved. The bone cancer pain model was prepared according to methods previously described (Yu et al., 2009). Briefly, right lateral superficial incisions were made in the skin covering the patella after disinfection with 75% v/v ethanol. A 23-gauge needle was inserted into the medullary cavity of the tibia at the intercondylar eminence 7 mm below the knee joint. The needle was removed and a 10 μ l microinjection syringe was used to inject either carcinoma cells (3.5×10^5) in 6 μ l PBS or 6 μ l PBS alone slowly into the right tibial cavity. The injection site was closed using bone wax after the syringe was removed; the wound was closed using 3-0 monofilament suture. After recovery, the animals were return to their home cage.

2.6. Paw withdrawal latency

The rats were tested for paw withdrawal latency (PWL) by a previously described method (Zhang et al., 2008). They were placed in a plastic chamber (25 cm $\times$ 15 cm $\times$ 15 cm) on the glass surface of the Full-Automatic Plantar Analgesia Tester (BME-410C, Chinese Academy of Medical Sciences, Institute of Biomedical Engineering, China) and allowed to acclimatize for 20 min before the test. PWL was measured pre-surgery and 4 h post-herbal application on days 6, 16, and 20 after injection of the Walker 256 cells. Mean PWL was established by averaging the latency of four tests with a 5-min interval between each test. Paws were alternated randomly to preclude “order” effects. A 20-s cut-off was used to prevent tissue damage. The investigator who performed the behavioral tests was blind to group assignment.

2.7. Mechanical threshold

The same groups of rats were used to test sensitivity to mechanical stimuli. Hind paw mechanical threshold was measured using von Frey filaments as described by Dixon. Testing was performed pre-surgery and 4 h post-herbal application on days 5, 11, 17, and 21. A set of von Frey filaments, hair-like instruments of logarithmically incremental stiffness (0.40, 0.60, 1.4, 2.0, 4.0, 6.0, 8.0, and 15.0 g; Stoelting Co, Wood Dale, Illinois,

USA), was used. Beginning with 2.0 g, a filament was applied to the mid-plantar surface of the hind paw, avoiding the less sensitive tori, and held perpendicularly about 1–2 seconds. A positive response was defined as a withdrawal of the paw upon stimulus. If a positive response occurred, the next lower von Frey hair was employed after a 10-minute delay; if a negative response occurred, the next higher hair was similarly applied. Five additional stimuli were given after the first response, and the pattern of response was converted to 50% von Frey threshold (Dixon, 1980).

2.8. Detection of serum ICTP and BAP

After treatment on days 0, 5, 11, 17, and 21, a blood sample was collected from the heart, and ICTP and BAP concentrations in serum were measured using commercial Enzyme-linked immunosorbent assay (ELISA) kits (Pierce Chemical, Rockford, IL, USA) according to the manufacturer's instructions.

2.9. Histological examination and identification of osteoclasts in bone

The rats were deeply anesthetized with pentobarbital at baseline and on day 21 after the cancer cell inoculation, then transcardially perfused with saline ($n=5$). Tissue from the right tibia of each animal was removed, preserved in 10% neutral buffered formalin, and decalcified in ethylenediaminetetraacetic acid (EDTA, 10%) for three weeks. The preserved tissues were embedded in paraffin, and 5-micron sections were cut and stained using the standard HE method. To identify osteoclasts and osteoblasts, tartarate-resistant acid phosphatase staining (tartarate-resistant acid phosphatase, TRAP staining kits, Nanjing, China), and alkaline phosphatase staining (alkaline phosphatase, AKP staining kits, Nanjing, China) were performed. Osteoclasts and osteoblasts were respectively counted on 10 sections from each rat, averaged for each rat, and then averaged for the group. Histopathological observation was conducted under a light microscope (Leica DM 2500), and the responsible investigator was blinded to animal treatment assignment.

2.10. Statistical analysis

The behavioral data was expressed as mean $\pm$ standard deviation (SD) and analyzed with repeated measure ANOVA; ELISA data were analyzed by one-way ANOVA followed by a Newman-Keuls test using SPSS 13.0 statistical software. Statistical significance in all analyses was considered to be $P < 0.05$.

3. Results

3.1. Adverse effects and toxicity

No abnormal animal behavioral changes or clinical signs were observed in rats treated with low or high dosage TLSJ gel, and their body weight, measured at baseline and on days 14, 28 and 42 after administration of the gel, was similar to that in the vehicle group (Figure 2). Heart, liver, and kidney function-related biochemistry tests showed no significant difference between treatment and vehicle groups (Tables 1, 2, and 3). Histological examination of heart, liver, spleen, kidney, and skin tissue of TLSJ-treated rats also showed no abnormal histological changes as compared to vehicle-treated rats (Figure 3).

3.2. TLSJ gel on pain

3.2.1. Histological evaluation of bone destruction—Histological examination showed no bone destruction in animals inoculated with PBS. In contrast, tumor growth and bone destruction were extremely severe on days 21 in animals that received the Walker 256 carcinoma cells (Figure 4). These results suggest that the cancer cells gradually destroyed the tibia.

3.2.2. Body weight and general health—The animals showed no marked weight loss 7 days after inoculation, but those that received the tumor cell inoculation showed slight weight loss compared to the sham group (PBS only) by day 11. After application of TLSJ gel for 21 days, the body weight of the rats in the treatment group increased significantly compared to that of those in the vehicle group (Figure 5).

3.2.3. Mechanical allodynia—Cancer cell-inoculated rats displayed a profound ($P < 0.05$) decrease of paw withdrawal threshold to von Frey hair stimulation on the paw ipsilateral to the cancer-cell inoculated tibia (Figure 6) on days 11–21 compared to PBS-inoculated rats, which showed no changes over 21 days. The TLSJ-treated rats displayed a significant ($P < 0.01$) increase in paw withdrawal threshold on days 17–21 compared to the vehicle group; the contralateral hind paw showed no significant changes. These data demonstrate that TLSJ significantly inhibited bone cancer-induced mechanical allodynia.

3.2.4. Thermal hyperalgesia—Figure 7 shows the effect of TLSJ gel on PWL. Compared to PBS-inoculated rats, cancer-inoculated rats displayed a profound ($P < 0.01$) decrease of PWL, an indication of thermal hyperalgesia, on the hind paw ipsilateral to cancer-inoculated tibia on days 10–20. TLSJ treatment significantly ($P < 0.01$) increased PWL on days 16–20 compared to vehicle control. The contralateral hind paw showed no significant changes in PWL. These data demonstrate that TLSJ significantly inhibited bone cancer-induced thermal hyperalgesia.

3.2.5. Serum ICTP and BAP levels and osteoclastic and osteoblastic activity—Serum ICTP and BAP values are presented in Figures 8 and 9. The values were elevated significantly ($P < 0.01$) and time-dependently in the cancer-inoculated rats compared to those inoculated with PBS. TLSJ treatment significantly ($P < 0.01$) hampered the increase of ICTP and BAP compared to vehicle control.

Osteoclasts and osteoblasts were significantly increased in tumor cell-bearing bone compared to PBS-injected bone (Figures 10 and 11). TLSJ treatment significantly decreased osteoclasts and osteoblasts, indicating an inhibition of osteoclastic and osteoblastic activity.

4. Discussion

In the present study, Chinese herbal TLSJ gel demonstrated a significant analgesic effect, decreasing both mechanical and thermal sensitivity, which correlate to progressive decreases in serum ICTP and BAP, during days 11–21 in the bone cancer pain of this rat model.

According to TCM theory, cancer pain is the result of tumor-caused blockage of Qi and blood flow through the channels of the body. This occurs in two phases. First, stagnation of Qi and blood causes pain. Second, the insufficiency of Qi and blood causes pain. Thus the main therapeutic goals are to promote Qi and activate blood, soften hard lumps and dispel nodes, replenish Qi and blood and warm the channels (Chen and Li, 2005). TLSJ, which significantly inhibited bone cancer-induced pain in this study, was formulated accordingly.

Consistent with our data, previous studies show that a number of the individual herbs in the TLSJ formula inhibit pain. For instance, *Trachelospermum jasminoides* significantly inhibited acetic acid-induced writhing responses and formalin-induced pain (Sheu et al., 2009). *Corydalis yanhuso* (rhizome), combined with *Angelicae dahuricae*, alleviated pain in a clinical trial (Yuan et al., 2004). *Boswellia carteri* (gum resin) significantly lengthened PWL in a inflammatory pain rat model (Fan et al., 2005), and *Commiphora myrrha* (resin) significantly reduced incidents of writhing in mice (Su et al., 2012). Additionally, an extract of *Melia toosendan* (fruit) showed significant analgesic effects in both writhing and hot-

plate tests (Xie et al., 2008). These literature reports lend further support to our observation that TLSJ gel significantly alleviates cancer pain. We observed that TLSJ treatment decreased mechanical pain between treatment days 17–21 and thermal pain between days 10–20 post-cancer cell inoculation. These findings demonstrate that TLSJ inhibits thermal pain earlier than it does mechanical pain, suggesting differences in these kinds of pain. This observation is consistent with a previous report that thermal and mechanical pain are underpinned by differential mechanisms (Paqueron et al., 2003).

Further, our data shows that TLSJ significantly and progressively decreased serum ICTP and BAP levels. BAP is produced in extremely high amounts in tumor-bearing patients and is therefore an excellent indicator of bone formation activity (Plebani et al., 1996). Serum ICTP, a product of the degradation of type I collagen by matrix metalloproteases, is strongly affected by processes active in myeloma bone disease (Abildgaard et al., 2003). The lower, TLSJ-induced levels of ICTP and BAP suggest that TLSJ inhibits bone metastasis, which is also evidenced by the data showing that TLSJ inhibits osteoclastic and osteoblastic activity. It has been reported that osteoprotegerin, a naturally secreted decoy receptor that inhibits osteoclast maturation and activity and induces osteoclast apoptosis, diminishes bone cancer pain (Luger et al., 2001). Further, bisphosphonate alendronate inhibits bone cancer pain and reduces the number of activated osteoclasts (Sevcik et al., 2004). These previous studies suggest that osteoclasts play an essential role in bone cancer pain. Since osteoclasts absorb bone by maintaining an acidic pH extracellular microenvironment (4.0–5.0) at the osteoclast-mineralized bone interface (Delaisse and Vaes, 1992), osteoclast-mediated bone remodeling is accompanied by the robust production of extracellular protons. At normal body temperature, this acidic environment can activate transient receptor potential cation channel subfamily V member 1 (TRPV1) in primary afferent fibers that innervate bone (Tominaga et al., 1998). Acute or chronic administration of a TRPV1 antagonist or disruption of the TRPV1 gene produces significant attenuation of both ongoing and movement-evoked nocifensive behaviors in a bone cancer model (Ghilardi et al., 2005). Together, these data suggest that TLSJ alleviates pain by decreasing osteoclast activity, which in turn down-regulates TRPV1 activation. However, the mechanisms by which TLSJ inhibits osteoclastic activity needs further investigation.

In recent years the medical community and public have been concerned about herbal product safety (White House Commission on Complementary and Alternative Medicine, 2002). During the 42-day repeat-dose study, no observable adverse effects were found, and sera biochemical examination and histology observation showed no toxic effects. These findings suggest that the TLSJ used in the present study will show no toxicity or adverse effects in clinic.

In conclusion, in the present study TLSJ significantly inhibited bone cancer-induced thermal and mechanical sensitization by inhibiting osteoclastic activity and showed no adverse effects during six weeks of application. Inadequate pain control for cancer patients remains a serious problem; a reported 50% of cancer patients do not receive adequate pain treatment (Cleary, 2007; Strang, 1998; Wilson et al., 2007), which badly impairs their quality of life. TLSJ appears to be safe and effective for treating cancer pain. Although the effective dosage in rats does not directly translate into an effective human dosage, the data from the present study nevertheless provides important information for determining potential dosages in future clinical studies.

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Abbreviation list

ALT	Alanine aminotransferase
ALB	Albumin
AKP	Alkaline phosphatase
AST	Aspartate aminotransferase
BUN	Blood urea nitrogen
BAP	Bone-specific alkaline phosphatase
CREA	Creatinine
CK	Creatine kinase
CK-MB	Creatine kinase-MB (M, muscle subunit; B, Brain type subunit)
CysC	Cystatin C
ELISA	Enzyme-linked immunosorbent assay
EDTA	Ethylenediaminetetraacetic acid
FDA	Food and Drug Administration
HE	Hematoxylin and eosin
HBDH	Hydroxybutyrate dehydrogenase
ICTP	Type I collagen telopeptides
LDH	Lactate dehydrogenase
PWL	Paw withdrawal latency
PBS	Phosphate buffered saline
SD	Standard deviation
TBIL	Total bilirubin
TCM	Traditional Chinese medicine
TLSJ	Tong-Luo-San-Jie
TP	Total protein
TRAP	Tartarate-resistant acid phosphatase
TRPV1	Transient receptor potential cation channel subfamily V member 1
UA	Uric acid
UPLC	Ultra performance liquid chromatography

References

- Abildgaard N, Brixen K, Kristensen JE, Eriksen EF, Nielsen JL, Heckendorff L. Comparison of five biochemical markers of bone resorption in multiple myeloma: elevated pre-treatment levels of S-ICTP and U-Ntx are predictive for early progression of the bone disease during standard chemotherapy. *British Journal of Haematology*. 2003; 120:235–242. [PubMed: 12542480]
- Adami S. Bisphosphonates in prostate carcinoma. *Cancer*. 1997; 80:1674–1679. [PubMed: 9362435]
- Banning A, Sjøgren P, Henriksen H. Pain causes in 200 patients referred to a multidisciplinary cancer pain clinic. *Pain*. 1991; 45:45–48. [PubMed: 1861877]

- Bensky, D.; Gamble, A. *Chinese Herbal Medicine: Materia Medica* (revised edition). Seattle, Washington: Eastland Press; 1993.
- Bowdle TA. Adverse effects of opioid agonists and agonist-antagonists in anaesthesia. *Drug Safety*. 1998; 19:173–189. [PubMed: 9747665]
- Chen T, Qing Z, Yu S. Clinical observation on 48 cases of cancer pain with Chinese medicine analgesic gelatin. *Chinese Journal of Information on TCM*. 2006; 13:7.
- Chen YZ, Li PP. Effectiveness and Application of Chinese Medicine for Cancer Pain. *Clinical Journal of Tradition Chinese Medicine*. 2005; 17:529–531.
- Cherny N, Ripamonti C, Pereira J, Davis C, Fallon M, McQuay H, Mercadante S, Pasternak G, Ventafridda V. Expert Working Group of the European Association of Palliative Care, N. Strategies to manage the adverse effects of oral morphine: an evidence-based report. *Journal of Clinical Oncology*. 2001; 19:2542–2554. [PubMed: 11331334]
- China State Administration of Traditional Chinese Medicine. *Testing Guidelines for Safety Evaluation of New Drug, The Guidelines for Developing New Chinese Herbal Medicine*. Beijing: China State Administration of Traditional Chinese Medicine; 1999.
- Cleary JF. The Pharmacologic Management of Cancer Pain. *Journal of Palliative Medicine*. 2007; 10:1369–1394. [PubMed: 18095817]
- Clohisey DR, Perkins SL, Ramnaraine ML. Review of cellular mechanisms of tumor osteolysis. *Clinical Orthopaedics & Related Research*. 2000;104–114. [PubMed: 10810467]
- Davies NM, Jamali F. COX-2 selective inhibitors cardiac toxicity: getting to the heart of the matter. *Journal of Pharmacy & Pharmaceutical Sciences*. 2004; 7:332–336. [PubMed: 15576013]
- Delaisse, J-M.; Vaes, G. Mechanism of mineral solubilization and matrix degradation in osteoclastic bone resorption. In: Rifkin, B.; Gay, C., editors. *Biology and physiology of the osteoclast*. MI: CRC, Ann Arbor; 1992. p. 289-314.
- Dixon WJ. Efficient analysis of experimental observations. *Annu Rev Pharmacol Toxicol*. 1980; 20:441–462. [PubMed: 7387124]
- Fan AY, Lao L, Zhang R, Wang L, Lee DY, Ma Z, Zhang W, Berman B. Effects of an acetone extract of *Boswellia carterii* Birdw. (Burseraceae) gum resin on rats with persistent inflammation. *Journal of Alternative & Complementary Medicine*. 2005; 11:323–331.
- FDA. Center for Drug Evaluation and Research (CDER), Guidance for Industry: Botanic drug products. 2004. www.fda.gov/cder/guidance/4592fnl.htm
- Gad, SC. *Drug Safety Evaluation*. New York: Wiley-Interscience; 2002.
- Ghilardi JR, Rohrich H, Lindsay TH, Sevcik MA, Schwei MJ, Kubota K, Halvorson KG, Poblete J, Chaplan SR, Dubin AE, Carruthers NI, Swanson D, Kuskowski M, Flores CM, Julius D, Mantyh PW. Selective blockade of the capsaicin receptor TRPV1 attenuates bone cancer pain. *Journal of Neuroscience*. 2005; 25:3126–3131. [PubMed: 15788769]
- Gozum S, Tezel A, Koc M. Complementary alternative treatments used by patients with cancer in eastern Turkey. *Cancer Nursing*. 2003; 26:230–236. [PubMed: 12832956]
- Gutgsell T, Walsh D, Zhukovsky DS, Gonzales F, Lagman R. A prospective study of the pathophysiology and clinical characteristics of pain in a palliative medicine population. *American Journal of Hospice and Palliative Medicine*. 2003; 20:140–148. [PubMed: 12693647]
- He L, Yang L, Xiong A, Zhao S, Wang Z, Hu Z. Simultaneous quantification of four indole alkaloids in *Catharanthus roseus* cell line C20hi by UPLC-MS. *Analytical Sciences*. 2011; 27:433–438. [PubMed: 21478621]
- Honore P, Luger N, Sabino M, Schwei M, Rogers S, Mach D, O'keefe P, Ramnaraine M, Clohisey D, Mantyh P. Osteoprotegerin blocks bone cancer-induced skeletal destruction, skeletal pain and pain-related neurochemical reorganization of the spinal cord. *Nature Medicine*. 2000; 6:521–528.
- Inturrisi CE. Management of cancer pain. *Pharmacology and principles of management*. *Cancer*. 1989; 63:2308–2320. [PubMed: 2566371]
- Koizumi M, Yamada Y, Takiguchi T, Nomura E, Furukawa M, Kitahara T, Yamashita T, Maeda H, Takahashi S, Aiba K. Bone metabolic markers in bone metastases. *Journal of Cancer Research & Clinical Oncology*. 1995; 121:542–548. [PubMed: 7559734]

- Lao, L. Essentials of Complementary and Alternative Medicine. In: Wayne, BJ.; Jeffery, SL., editors. Traditional Chinese Medicine, Part III. Philadelphia: Lippincott Williams & Wilkins; 1999. p. 216-232.
- Li RW, David Lin G, Myers SP, Leach DN. Anti-inflammatory activity of Chinese medicinal vine plants. *Journal of Ethnopharmacology*. 2003; 85:61–67. [PubMed: 12576203]
- Liu J, Han Y, Yang M, Zhu Q, Hu J. Preparation of paeonol microemulsion gel its and transdermal characterization in vitro. *China Journal of Chinese Materia Medica*. 2009; 34:2730–2733. [PubMed: 20209902]
- Luger NM, Honore P, Sabino MA, Schwei MJ, Rogers SD, Mach DB, Clohisy DR, Mantyh PW. Osteoprotegerin diminishes advanced bone cancer pain. *Cancer Research*. 2001; 61:4038–4047. [PubMed: 11358823]
- Pargeron KL, Hailey BJ. Barriers to effective cancer pain management: a review of the literature. *Journal of Pain & Symptom Management*. 1999; 18:358–368. [PubMed: 10584460]
- Paqueron X, Conklin D, Eisenach JC. Plasticity in action of intrathecal clonidine to mechanical but not thermal nociception after peripheral nerve injury. *Anesthesiology*. 2003; 99:199–204. [PubMed: 12826860]
- Pasternak GW. Multiple morphine and enkephalin receptors and the relief of pain. *JAMA*. 1988; 259:1362–1367. [PubMed: 2828704]
- Plebani M, Bernardi D, Zaninotto M, De Paoli M, Secchiero S, Sciacovelli L. New and traditional serum markers of bone metabolism in the detection of skeletal metastases. *Clinical Biochemistry*. 1996; 29:67–72. [PubMed: 8929827]
- Radha Krishna LK, Poulouse JV, Tan BS, Goh C. Opioid use amongst cancer patients at the end of life. *Annals of the Academy of Medicine, Singapore*. 2010; 39:790–797.
- Richardson MA, Sanders T, Palmer JL, Greisinger A, Singletary SE. Complementary/alternative medicine use in a comprehensive cancer center and the implications for oncology. *Journal of Clinical Oncology*. 2000; 18:2505–2514. [PubMed: 10893280]
- Sareen R, Kumar S, Gupta GD. Meloxicam carbopol-based gels: characterization and evaluation. *Current Drug Delivery*. 2011; 8:407–415. [PubMed: 21453256]
- Schaffer D, Florin T, Eagle C, Marschner I, Singh G, Grobler M, Fenn C, Schou M, Curnow KM. Risk of serious NSAID-related gastrointestinal events during long-term exposure: a systematic review. *Medical Journal of Australia*. 2006; 185:501–506. [PubMed: 17137455]
- Scheiman JM. The impact of nonsteroidal anti-inflammatory drug-induced gastropathy. *American Journal of Managed Care*. 2001; 7:S10–S14. [PubMed: 11225347]
- Sevcik MA, Luger NM, Mach DB, Sabino MAC, Peters CM, Ghilardi JR, Schwei MJ, Röhrich H, De Felipe C, Kuskowski MA, Mantyh PW. Bone cancer pain: the effects of the bisphosphonate alendronate on pain, skeletal remodeling, tumor growth and tumor necrosis. *Pain*. 2004; 111:169–180. [PubMed: 15327821]
- Sheu MJ, Chou PY, Cheng HC, Wu CH, Huang GJ, Wang BS, Chen JS, Chien YC, Huang MH. Analgesic and anti-inflammatory activities of a water extract of *Trachelospermum jasminoides* (Apocynaceae). *Journal of Ethnopharmacology*. 2009; 126:332–338. [PubMed: 19703536]
- Smith MR. Markers of bone metabolism in prostate cancer. *Cancer Treatment Reviews* 32 Suppl. 2006; 1:23–26.
- Strang P. Cancer pain--a provoker of emotional, social and existential distress. *Acta Oncologica*. 1998; 37:641–644. [PubMed: 10050980]
- Su S, Hua Y, Wang Y, Gu W, Zhou W, Duan J-a, Jiang H, Chen T, Tang Y. Evaluation of the anti-inflammatory and analgesic properties of individual and combined extracts from *Commiphora myrrha*, and *Boswellia carterii*. *Journal of Ethnopharmacology*. 2012; 139:649–656. [PubMed: 22178177]
- Tamada T, Sone T, Tomomitsu T, Jo Y, Tanaka H, Fukunaga M. Biochemical markers for the detection of bone metastasis in patients with prostate cancer: diagnostic efficacy and the effect of hormonal therapy. *Journal of Bone & Mineral Metabolism*. 2001; 19:45–51. [PubMed: 11156473]
- Tominaga M, Caterina MJ, Malmberg AB, Rosen TA, Gilbert H, Skinner K, Raumann BE, Basbaum AI, Julius D. The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron*. 1998; 21:531–543. [PubMed: 9768840]

- van den Beuken-van Everdingen M, de Rijke J, Kessels A, Schouten H, van Kleef M, Patijn J. Prevalence of pain in patients with cancer: a systematic review of the past 40 years. *Annals of Oncology*. 2007; 18:1437–1449. [PubMed: 17355955]
- Virik K, Glare P. Pain management in palliative care. Reviewing the issues. 2000; 29:1027–1033.
- Wang C, Li Y, Gao J, He Y, Xiong A, Yang L, Cheng X, Ma Y, Wang Z. The comparative pharmacokinetics of two pyrrolizidine alkaloids, senecionine and adonifoline, and their main metabolites in rats after intravenous and oral administration by UPLC/ESIMS. *Analytical & Bioanalytical Chemistry*. 2011; 401:275–287. [PubMed: 21573843]
- White House Commission on Complementary and Alternative Medicine. White House Commission on Complementary and Alternative Medicine Policy. 2002. <http://www.whccamp.hhs.gov/finalreport.html>
- Wilson KG, Chochinov HM, McPherson CJ, LeMay K, Allard P, Chary S, Gagnon PR, Macmillan K, De Luca M, O'Shea F, Kuhl D, Fainsinger RL. Suffering With Advanced Cancer. *Journal of Clinical Oncology*. 2007; 25:1691–1697. [PubMed: 17470861]
- Xie F, Zhang M, Zhang C-F, Wang Z-T, Yu B-Y, Kou J-P. Anti-inflammatory and analgesic activities of ethanolic extract and two limonoids from *Melia toosendan* fruit. *Journal of Ethnopharmacology*. 2008; 117:463–466. [PubMed: 18384989]
- Xu L, Lao LX, Ge A, Yu S, Li J, Mansky PJ. Chinese herbal medicine for cancer pain. *Integrative Cancer Therapies*. 2007; 6:208–234. [PubMed: 17761635]
- Yu S, Peng HD, Ju DW, Wei PK, Xu L, Lao LX, Li J. Mechanisms of treatment of cancer pain with a topical Chinese herbal formula in rats. *Chinese Medical Journal*. 2009; 122:2027–2031. [PubMed: 19781391]
- Yuan C-S, Mehendale SR, Wang C-Z, Aung HH, Jiang T, Guan X, Shoyama Y. Effects of *Corydalis yanhusuo* and *Angelica dahurica* on Cold Pressor-Induced Pain in Humans: A Controlled Trial. *The Journal of Clinical Pharmacology*. 2004; 44:1323–1327.
- Zhang RX, Li A, Liu B, Wang L, Xin J, Ren K, Qiao JT, Berman BM, Lao L. Electroacupuncture attenuates bone-cancer-induced hyperalgesia and inhibits spinal preprodynorphin expression in a rat model. *European Journal of Pain*. 2008; 12:870–878. [PubMed: 18221900]

Figure 1. UPLC profile of TLSJ gel
Charateristic peaks are identified by their retention time.

Figure 2. Change in body weight of rats treated with TLSJ gel or inert gel for 42 days (n=10 per group)

Body weight, measured on days 0 (baseline), 14, 28, and 42 after administration, showed normal increase over the 42 days. There was no significant difference among the three groups of rats.

Figure 3. Microphotographs of tissue histology of rats treated with TLSJ or inert gel for 42 days (HE staining, original magnification: 400×)
 A: TLSJ gel, 0.5 g/cm²/day. B: TLSJ gel, 1 g/cm²/day. C: Inert gel. 1: Spleen. 2: Liver. 3: Kidney. 4: Heart. 5: Skin. The histology is similar in the three groups of rats, and no tissue damage was found.

Figure 4. Histological evaluation of bone destruction in inert gel-treated rats
Histology of tibial destruction at days 0 (A) and 21 (B) after carcinoma cell injection.
Cancer cells are clustered densely in the marrow. Original magnification: 400×. The inserts
are higher-magnification of corresponding boxes. Arrows point to cancer cells.

Figure 5. Change in body weight of rats with cancer cells + TLSJ gel (Treatment), cancer cell + inert gel (Vehicle) or PBS inoculation (Sham)

Body weight was measured on days 0 (baseline), 5, 11, 17, and 21 after cancer cell inoculation into the tibia. ** $P < 0.01$ vs sham group; *** $P < 0.01$ vs vehicle group, $n = 10$.

Figure 6. Changes in mechanical allodynia (mean $\pm$ SE, n=10 per group)

Mechanical allodynia was measured on days 0 (baseline), 5, 11, 17, and 21 after surgery. Cancer induced a significant decrease of mechanical threshold of the hind paw ipsilateral to the cancer-inoculated tibia; TLSJ gel treatment significantly restored it compared to inert gel control. Contralateral mechanical allodynia showed no changes. **P<0.01 vs sham group; #P <0.05, ##P <0.01 vs vehicle group.

Figure 7. Changes in thermal hyperalgesia (mean $\pm$ SE, n=10 per group)

Thermal hyperalgesia was measured on days 0 (baseline), 4, 10, 16, and 20 after surgery. Cancer induced a significant decrease of paw withdrawal latency (PWL) of the hind paw ipsilateral to the cancer cell-inoculated tibia. TLSJ gel treatment significantly restored it compared to inert gel control. Contralateral paws showed no change in PWL. **P<0.01 vs sham group, ##P <0.01 vs vehicle group.

Figure 8. Serum ICTP levels (mean $\pm$ SD, $n=5$ per group)

Concentrations of serum ICTP were measured with ELISA on days 0 (baseline), 5, 11, 17, and 21 after surgery. ** $P<0.01$ vs sham group, ### $P<0.01$ vs vehicle group.

Figure 9. Serum BAP levels (mean $\pm$ SD, $n=5$ per group)

Concentrations of serum BAP were measured with ELISA on days 0 (baseline), 5, 11, 17, and 21 after surgery. ** $P<0.01$ vs sham group, ### $P<0.01$ vs vehicle group.

Figure 10.

A: Photographs of a tartarate-resistant acid phosphatase stained tibia. Micrographs were taken from the tibia on days 0 (a, c, and e) and 21 (b, d, f) after inoculation with 3.5×10^5 of Walker 256 rat mammary gland carcinoma cells: a and b are sham rats; c and d are inert gel treated-rats; e and f are TLSJ gel-treated rats. Arrows point to osteoclasts. **B: Osteoclast values in the three groups of rats.** Note that tumors induced significant higher osteoclast staining compared to sham, and TLSJ gel treatment significantly decreased the staining. The inserts are higher-magnification of corresponding boxes.

Figure 11.

A: Photographs of an alkaline phosphatase-stained tibia. Micrographs were taken from the tibia on days 0 (a, c, and e) and 21 (b, d, f) after inoculation with 3.5×10^5 of Walker 256 rat mammary gland carcinoma cells: a and b are sham rats; c and d are inert gel-treated rats; e and f are TLSJ gel-treated rats. Arrows point to osteoblasts. **B: Osteoblast values in the three groups of rats.** Note that tumors induced significantly higher osteoblast staining compared to sham, and TLSJ gel treatment significantly decreased the staining. The inserts are higher-magnification of corresponding boxes.

Table 1

Effect of TLSJ treatment on serum myocardial enzymes in rats

Group	N	CK (IU/L)	LDH (U/L)	CK-MB (IU/L)	HBDH (IU/L)
Low dosage	10	1004.15 ± 119.51	261.21 ± 16.83	359.73 ± 69.59	268.05 ± 22.17
High dosage	10	1033.69 ± 109.01	275.38 ± 32.20	387.19 ± 59.34	271.18 ± 18.15
Inert gel	10	1007.23 ± 125.21	266.60 ± 24.20	360.77 ± 56.97	278.40 ± 16.81

Table 2

Effect of TLSJ treatment on serum liver function-related enzymes in rats

Group	N	TP (g/L)	ALB (g/L)	ALT (U/L)	AST (U/L)	TBIL (μmol/L)
Low dosage	10	57.66 ± 5.69	40.75 ± 6.61	41.93 ± 6.22	157.98 ± 36.28	1.38 ± 0.24
High dosage	10	55.25 ± 3.32	40.80 ± 2.33	45.12 ± 10.21	143.26 ± 43.21	1.48 ± 0.25
Inert gel	10	56.00 ± 4.40	40.92 ± 2.90	44.47 ± 3.27	155.10 ± 34.43	1.42 ± 0.24

Table 3

Effect of TLSJ treatment on serum kidney function-related metabolites in rats

Group	N	CREA (nmol/L)	BUN (mmol/L)	UA (μmol/L)	CYSC (mg/L)
Low dosage	10	29.84 ± 2.19	6.58 ± 0.93	69.15 ± 8.69	0.36 ± 0.04
High dosage	10	30.62 ± 5.03	7.42 ± 0.99	70.60 ± 5.87	0.34 ± 0.03
Inert gel	10	31.62 ± 2.59	6.83 ± 1.11	70.31 ± 14.58	0.33 ± 0.02