Involvement of Substance P/Neurokinin-1 Receptor in the Analgesic and Anticancer Activities of Minimally Toxic Fraction from the Traditional Chinese Medicine Liu-Shen-Wan in Vitro

Xiao-Jun Li, a,b Mei-Mei Jia, a,# Yu-Sang Li, a,* Yan-Ling Yang, a Xian-Qing Mao, b and He-Bin Tang a

a Department of Pharmacology, College of Pharmacy, South-Central University for Nationalities: Wuhan 430074, China; and b Laboratory of Molecular and Cellular Oncology, Department of Oncology, Public Research Center for Health (CRP-Santé); 84, Val Fleur; L-1526, Luxembourg.

Received October 9, 2013; accepted December 15, 2013; advance publication released online December 21, 2013

Liu-Shen-Wan (LSW), an ancient preparation used to treat localized infection with pain, was recently reported to possess anticancer activity. The mechanism responsible for LSW’s analgesic and anticancer activity is unclear. In the present study, we obtained a LSW supernatant (LSWS) fraction from ultrasound-assisted ethanol extraction (yield 15.9%) which proved to be safer than LSW in terms of hepatotoxicity. The LSWS (1 and 10 µg/mL) exhibited a potent inhibitory effect on the bradykinin-evoked rapid release of substance P from dorsal root ganglion (DRG) cells. At concentrations of 0.1 µg/mL and higher, the LSWS resulted in a concentration-related growth inhibitory effect on HepG2, a representative cancer cell line. The LSWS significantly down-regulated the neurokinin-1 (NK-1) receptor expression in both HepG2 and bradykinin-treated DRG cells. In addition to the NK-1 receptor-dependent growth inhibition in HepG2 cells (0.1–100 µg/mL), the LSWS induced mitochondria-mediated apoptosis at a higher concentration (1–100 µg/mL). In conclusion, we recently isolated a safer LSW fraction which maintained its analgesic and anticancer activity, and found that the substance P/NK-1 receptor system was partly responsible for these effects. Our findings will be useful for developing more effective and less toxic LSW preparations.

Key words Liu-Shen-Wan; anticancer; analgesic; substance P; neurokinin-1 receptor

Liu-Shen-Wan (LSW), a well-known heat clearing and detoxifying herbal preparation, is mainly composed of Calculus Bovis Cowherb Seed, Margarita Pecavine, Venenum Bufonis Toborinone, Broneolum Syntheticum Boro-Scopol, Moschus Muskone, and Realgar Reapam. This ancient prescription is effective for treating localized infections and inflammation-associated pain. LSW’s analgesic effect has been known and used for more than 200 years. During the past 200 years, many other applications have been uncovered, including its use for the treatment of diphtheria, scarlet fever, pharyngotonsillitis, acute tonsillitis, purulent parotitis, encephalitis B and viral pneumonia. It was also recently reported that LSW had a potential anticancer activity.

Despite its approval by the China Food and Drug Administration and its use for nearly 200 years, traditional LSW is associated with some toxicity because it contains realgar and Venenum Bufonis, both of which were found to be toxic if administered alone. This toxicity made LSW unacceptable to the international community as a therapeutic agent. In the present study, we isolated an ethanol fraction of LSW which maintained its analgesic and anticancer activities, but with a decreased level of toxicity. However, to the best of our knowledge, the mechanism(s) underlying LSW’s analgesic and anticancer activity are still unclear.

Because LSW has both analgesic and anticancer activities, we suppose that there might be some common mechanisms underlying these two activities. Substance P and its endogenous receptor, the neurokinin-1 (NK-1) receptor, which play important roles in both pain transmission and tumor formation, were hypothesized to be involved in these activities. Substance P is an important element in pain perception. The sensory function of substance P is thought to be related to the transmission of pain information into the central nervous system. Moreover, substance P and the NK-1 receptor have recently been discovered to play an integral role in the maintenance of a favorable tumor microenvironment. After binding to the NK-1 receptor, substance P also regulates biological functions related to cancer, including tumor cell proliferation (favoring tumor growth), angiogenesis, and the migration of the tumor cells for invasion and metastasis. In addition, it was demonstrated that NK-1 receptor blockers could inhibit the growth of tumor cells.

Thus, we hypothesized that the substance P/NK-1 receptor system may be involved in both the analgesic and anticancer activities of LSW. In an attempt to explore this hypothesis, we measured the levels of substance P, the NK-1 receptor and other related factors in LSW-treated cancer cell lines and dorsal root ganglion (DRG) cells.

MATERIALS AND METHODS

Preparation of LSW Fractions The LSW used in this study was purchased from Shanghai Leiyunshang Pharmaceutical Co., Ltd., China. LSW (Lot No.: 090808, 500 mg) was milled and suspended in 5 mL ethanol for 3 h. This was followed by ultrasound-assisted extraction for 30 min (50 kHz), centrifugation at 1000×g for 15 min and filtration (0.45 µm filter) of the supernatant. Then, the residue and the supernatant were lyophilized and stored at 4°C until further use (Fig. 1). The yields of the LSW supernatant (LSWS) and LSW residue (LSWR) were 15.9% and 74.3%, respectively. There was material loss during the filtration of the supernatant of LSW.

LC-MS/MS Analysis Identification of the major compo-

The authors declare no conflict of interest.

# These authors contributed equally to this work.

* To whom correspondence should be addressed. e-mail: liys2006@mail.scuec.edu.cn

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The LSWS was performed by LC-MS/MS on a U3000-Dionex and Thermo LCQ FLEET ion Trap MS instrument with a 5 µm Alltima C-18 column (4.6×250 mm) at 30°C. Detection was carried out at 296 nm with a 40-min gradient. Mass spectra were recorded using electrospray ionization in positive mode with an active focus, scanning from 100 to 1000 m/z. Bufotenidine, cinobufotalin, bufotalin, bufalin, cinobufagin, and resibufoenin were identified in the LSWS.

In Vivo Toxicity and Safety Evaluation

Standard dose of LSW for a 60 kg person is 0.52 mg/kg. However, some doctors often describe a single dose of 7.82 mg/kg LSW for patient with sore throat, which is the maximum dose used in clinical practice. To convert the dose used in humans to a dose based on surface area for mice, multiply 7.82 mg/kg by the Ks factor (37) for a human and then divide by the Km factor (3) for a mouse. This calculation results in a human equivalent dose for LSW of 96.4 mg/kg. For in vivo toxicity and safety evaluation, animals received a single oral dose of 964 mg/kg. Male Kunming mice (weighing 20–24 g) were purchased from the experimental animal research center of Hubei Province (Hubei, Wuhan, China). All experiments followed WHO Guidance of Humane Care and Use of Laboratory Animals. Forty animals were randomly divided into four groups of 10 animals each: group 1 was the control group; group 2 received LSW; group 3 received LSWS and group 4 received LSWR. The LSW, LSWS and LSWR were suspended in saline. The animals in the control group were administered an equal volume of saline orally. Two weeks later, the animals were sacrificed, and we found that liver was the only organ which showed obvious changes. The livers were harvested, fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 µm and stained with hematoxylin–eosin (H&E) for a histological analysis.

Cell Culture

Human hepatoma HepG2 cells and normal human liver L02 cells were purchased from China Center for Type Culture Collection (CCTCC, Wuhan, China) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Sigma, Louis, MO, U.S.A.), 200 µM L-glutamine and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, U.S.A.) at 37°C in a water-saturated atmosphere containing 5% CO2. DRGs of adult Wistar rats (6–9 weeks of age) were dissociated into single isolated neurons and non-neuronal cells by enzymatic treatment (collagenase and trypsin) according to a previously described method. Next the cells were plated on polyethyleneimine and laminin-coated cover glasses and incubated in DMEM containing 10% heat-inactivated horse serum. DRG
Flow Cytometric Analysis of Cells

**Flow Cytometric Analysis of HepG2**

Flow cytometric analyses were performed to determine the extent of HepG2 apoptosis, using an Annexin-V and propidium iodide (PI) double staining kit according to the manufacturer’s instructions. Briefly, 5×10^5 cells were cultured with or without the LSWS (0.1, 1, 10, and 100 µg/mL) for 24 h. Cells were then stained with Annexin V-fluorescein isothiocyanate (V-FITC) and propidium iodide (PI) double staining kit according to the manufacturer’s instructions. Brieﬂy, 5×10^5 cells were cultured with or without the LSWS for 24 h. Cells were then stained with Annexin V-FITC and PI in Hans’ balanced salt solution. The samples were subsequently analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.).

**Cell Viability Assay for HepG2 and L02 Cells**

Hepatoma cell line HepG2 and normal human liver cell line L02 were used for cell viability assay. Cells (1×10^5 cells) were added to each well of a 96-well plate and incubated overnight under serum-free conditions. Next, the cells were cultured in the presence or absence of various concentrations (0.1, 1, 10, and 100 µg/mL) of LSW, LSW residue (LSWR) or LSWS for 24 h. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to assess the viability of the HepG2 cells as described previously. Doxorubicin, a common chemotherapeutic drug, was used as a positive control.

Measurement of the Substance P Content from the DRG

**Cell Culture Medium**

Substance P is an important neurotransmitter released from primary afferent neurons that conveys nociceptive information and transmits pain signals. To clarify the possible pharmacological mechanism of LSWS on the modulation of responses to pain, we examined the effects of LSWS on the release of substance P by cultured DRG cells following treatment with bradykinin. The cells were pretreated with LSWS (for 15 min) prior to exposure to bradykinin. Except for cells that belonged to the control group, all other cultured cells were exposed to bradykinin (10 µM) or to both bradykinin (100 µM) and LSWS (10 µg/mL) for 3 h. The substance P content in the culture medium was measured by a highly sensitive radioimmunoassay as described previously.

**Immunocytochemical Staining of the NK-1 Receptor in DRG Cells**

The most important endogenous receptor for substance P is the NK-1 receptor. The binding of substance P to the NK-1 receptor has been associated with the transmission of pain signal. Therefore, following the substance P assay above, we further evaluated the effects of LSWS on NK-1 receptor expression. Immunocytochemical staining of the NK-1 receptor in DRG neurons cultured on cover glasses was performed using a standard immune-peroxidase technique according to the manufacturer’s instructions. Briefly, 4% paraformaldehyde-fixed cultured DRG cells on cover-glasses were incubated with goat serum (1:9 dilution, Sigma) for 1 h at room temperature. Then, an anti-NK-1 antibody (1:1000 dilution, Sigma) was added to the cells at 4°C overnight. After being washed with phosphate buffered saline (PBS), Alexa Fluor 546 (1:1000 dilution, Sigma) was added, and cells were incubated for 1 h at room temperature. Finally, the fluorescence signals were visualized with a microscope (Nikon Eclipse Ti fluorescent microscope, Japan), and images were captured by a CCD camera.

**Western Blotting Analysis**

Cells grown in 35 mm dishes were disrupted, and the protein concentration was determined by the Lowry method. From each sample, 20 µg of protein was separated by electrophoresis on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels, and then the proteins were electrothermally transferred to a polyvinylidylenefluoride (PVDF) membrane. The blots were incubated with specific primary antibodies (mouse anti-β-actin, rabbit anti-NK-1 receptor (both, 1:1000 dilution); rabbit anti-caspase 3, rabbit anti-caspase 8, rabbit anti-caspase 9, rabbit anti-Bcl-2, and rabbit anti-Bax antibodies (all, 1:300 dilution)) overnight at 4°C, and then were further incubated with a secondary antibody (rabbit polyclonal anti-immunoglobulin G (IgG), mouse monoclonal anti-IgG (both, 1:2000 dilution)) for 1 h at room temperature. Antibody binding was detected by using an enhanced chemiluminescence kit with hyper-enhanced chemiluminescence (ECL) film. The antibodies were purchased from Boster (Wuhan, China).

**Statistical Analysis**

The data were expressed as the means±S.E.M. The statistical analysis was performed with the SPSS statistical software program for Microsoft Windows, release 16.0 (Professional Statistic, Chicago, IL, U.S.A.). The data were analyzed using a one-way ANOVA with Dunnett’s multiple comparisons, with p values <0.05 considered to be statistically significant. IC_{50} values were calculated with GraphPad Prism 5.0 (GraphPad Software, Inc., CA, U.S.A.), using a nonlinear regression.

**RESULTS**

**Acute Hepatotoxicity of the LSWS in Mice**

Representative histopathology photomicrographs are shown in Fig. 3. The single high dose of LSW or its residue produced widespread damage to the liver resulting in cellular death and a loss of the normal liver structure. In comparison, the livers in the LSWS-treated group did not show morphological alterations. As mentioned above, the dose of 964 mg/kg was selected based on the typical Chinese doctor’s prescription for a sore throat. This dose was less than half of the 2000 mg/kg dose used in the limited testing. A standard protocol will be needed in the
Effects of the LSWS on the Apoptosis of Tumor Cell Lines

Both LSW and the LSWS produced a dose-related growth inhibitory effect on HepG2 cells (Fig. 4). Compared with control, LSW (1–100 µg/mL) and LSWS (0.1–100 µg/mL) treatments induced significant decrease of cell viability ($p < 0.001$). As for LSWR, it increased slightly the cell viability at lower concentrations (0.1–1 µg/mL), but significantly decreased of cell viability at higher concentrations ($p < 0.001$ vs. control).

The positive control doxorubicin showed cytotoxicity both in HepG2 and L02 cells, with the IC$_{50}$ 0.27 and 1.04 µg/mL, respectively. The IC$_{50}$ for L02 cells is about 4-fold of that for HepG2. However, LSWS’s performance is better than doxorubicin. Its IC$_{50}$ for L02 cells (62.58 µg/mL) is about 12-fold of that for HepG2 (5.54 µg/mL). At 10 µg/mL concentration, LSWS inhibited >40% HepG2, but just <10% L02. That
indicated that LSWS has relative selective cytotoxicity to hepatoma cell line to a certain degree. To test whether the cell death was a result of apoptosis in HepG2 cells, a flow cytometric analysis was performed using Annexin V and PI. Early stage apoptotic cells will only take up the Annexin V stain but will remain PI negative, and late-stage apoptotic cells will be positive for both Annexin V and PI. The results indicated that there were very few PI-positive and Annexin-V-positive cells in the control cultures (Fig. 5A, top first panel), while 23–44% of the LSWS-treated cells were labeled (lower right and upper right quadrants representing early and late apoptosis, respectively) in a concentration-dependent manner. After exposure of the cells to 0.1 µg/mL of the LSWS for 24 h, a significant number of cells showed Annexin V-FITC positive and PI negative staining (an increase in the number of dots in the bottom right quadrant from 0% of control cells to 17% of treated cells) (Fig. 5B). Following an increase of the LSWS to 100 µg/mL, the number of cells in advanced apoptosis (stained positive with both Annexin V-FITC and PI; upper right quadrant) was significantly increased from 6% of control cells to 31% of the treated cells (Figs. 5B–E).

The Effect of LSWS on the Bradykinin-Induced Substance P Release from Cultured DRG Cells

To clarify the possible pharmacological mechanism underlying the effects of the LSWS on the behavior responses to pain, we examined the effects of the LSWS on the release of substance P by cultured DRG cells induced by bradykinin treatment. As shown in Fig. 6, when the LSWS (10 µg/mL) was used alone, it had no significant effect on the substance P release from normal cultured DRG cells. However, the LSWS treatment (1 and 100 µg/mL) led to a potent inhibitory effect (159.3±13.2, 110.6±9.3 pg/dish, respectively) on the bradykinin-evoked rapid re-

Fig. 6. The Effect of the Liu-Shen-Wan Supernatant (LSWS) on the Substance P Release from Rat Dorsal Root Ganglion (DRG) Cells

Except for the cells in the control group, all of the other cultured cells were exposed to bradykinin (10 µM) or to both bradykinin (10 µM) and the LSWS (10 µg/mL) for 3 h. The substance P content in the collected culture medium was measured by a radioimmunoassay. The values are the means±S.E.M. of six replicates. *p<0.05, **p<0.01, and ***p<0.001.

Fig. 7. The Effect of the Liu-Shen-Wan Supernatant (LSWS) on the Neurokinin 1 (NK-1) Receptor Expression in Rat Dorsal Root Ganglion (DRG) Cells

Compared with control cells (A), the NK-1 receptor expression was significantly up-regulated by bradykinin treatment (B). Treatment with the LSWS (10 µg/mL) could significantly down-regulate the bradykinin-induced expression of the NK-1 receptor (C). (D) The relative levels were analyzed by determining the optical density of fluorescence signals. The 4% paraformaldehyde-fixed cultured DRG cells on cover-glasses were incubated with goat serum (1:9 dilution) for 1 h at room temperature. Then, an anti-NK-1 antibody (1:1000 dilution) was added to the cells, which were incubated at 4°C overnight. After being washed with PBS, Alexa Fluor 546 (1:1000 dilution; Sigma) was added, and cells were incubated for another 1 h at room temperature. Finally, the fluorescence signals were visualized with a microscope. Scale bar, 10 µm. The values are the means±S.E.M. of six replicates. ***p<0.001 vs. control, and ###p<0.001 vs. bradykinin-treated group.
Cells grown in 35 mm dishes were disrupted. A total of 20 µg of protein were separated by electrophoresis on 12% SDS-polyacrylamide gels, and then the proteins were electrophoretically transferred to PVDF membranes. Antibody binding was detected by using an enhanced chemiluminescence kit with hyper-ECL film. (A) Representative Western blots of the NK-1 receptor and β-actin expression. (B) The relative levels were analyzed by determining the ratio of the NK-1 receptor/β-actin. The LSWS (0.1–100 µg/mL) treatment significantly decreased the expression of the NK-1 receptor in HepG2 cells compared with the control cells. The values are the means±S.E.M. of three replicates. *p<0.05, **p<0.01, and ***p<0.001 vs. control.

**DISCUSSION**

Traditional LSW has been used clinically for more than 250 years, and increasing attention is being paid to its scientific evaluation. A previous study demonstrated that realgar-containing LSW (200 mg/kg) was much less acutely toxic than sodium arsenite and arsenate in mouse livers.5) Unfortunately, when the dose of such a drug was increased to 964 mg/kg (nearly five-fold the 200 mg/kg dose), it induced severe liver injury in mice. The slag following LSW alcohol solution (LSWR) also destroyed animal livers in experimental studies. However, the supernatant of LSW ethanol extraction (LSWS) seemed to be safer in the present study. Using the supernatant fraction. However, the possibility still exists that arsenic bioaccumulation may lead to the development of toxic effects over time, even when the amount present is not sufficient to produce an acute effect in a single dose study. Therefore, a study on the long-term toxicity will be necessary to confirm the safety of LSWS in the future.
Moreover, these findings are in agreement with previously due to NK-1 receptor antagonist pharmacological profile.

In conclusion, we have isolated a safer LSW fraction which maintained the analgesic and anticancer activity of LSW, but with reduced hepatotoxicity. We also found that the substance P/NK-1 receptor system was involved in both the analgesic and anticancer activities of this fraction. The present findings will be useful to develop more effective and less toxic preparations of LSW that will be acceptable to the international community.

Acknowledgments This work was supported by National Natural Science Foundation of China (81373842 and 81101538), Natural Science Foundation of China Hubei (2012FFC13501) and Grants from South-Central University for Nationalities (CZQ11037 and XTZ10001).

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