The Ethical Kampo Formulation Sho-Seiryu-To (TJ-19) Prevents Bleomycin-Induced Pulmonary Fibrosis in Rats

Chang-qing YANG, a,1) Peng-yuan SUN, a Da-zhi DING, b Hiroshi MORIUCHI, c Yoichi ISHITSUKA, * d Mitsuru IRIKURA, d and Tetsumi IRIE d e

a Department of Pharmaceutical Sciences, College of Pharmacy, Yanbian University; b Department of Cardiology, Affiliated Hospital of Yanbian University; Jilin Prov., Yanji 133000, China; c Laboratory of Pharmacy Practice, Faculty of Pharmaceutical Sciences, Sojo University; 4–22–1 Ikeda, Kumamoto 860–0082, Japan; d Department of Clinical Chemistry and Informatics, Graduate School of Pharmaceutical Sciences, Kumamoto University; and e Center for Clinical Pharmaceutical Sciences, Faculty of Pharmaceutical Sciences, Kumamoto University; 5–1 Oc-hommachi, Kumamoto 862–0973, Japan. Received February 15, 2010; accepted May 26, 2010; published online May 28, 2010

The effects of Sho-seiryu-to (TJ-19), an ethical Kampo formulation, on bleomycin (BLM)-induced pulmonary fibrosis in rats was examined. Pulmonary fibrosis was induced by intratracheal instillation of a single dose of BLM (5 mg/kg). The TJ-19 used consisted of at least 21 constituents, as determined by three-dimensional HPLC analysis, and was administered orally twice a day at a dose of 1.5 g/kg until the end of the study period. Changes in general appearance and body weight were monitored. Twenty-eight days after BLM instillation, the animals were sacrificed and the study parameters were measured. TJ-19 attenuated the loss in body weight, increase in lung/body weight ratio and concentration of hydroxyproline and malondialdehyde in the lung tissues induced by BLM administration. TJ-19 also prevented BLM-induced fibrotic changes in the lung histology. These protective effects of TJ-19 were observed when administration was started 1 week before and simultaneously with the instillation of BLM. These results suggest that TJ-19 has prophylactic potential against BLM-induced pulmonary fibrosis, and may therefore be a promising drug candidate and medicinal resource for preventing BLM-induced and idiopathic pulmonary fibrosis.

Key words TJ-19; lung injury; pulmonary fibrosis; bleomycin; oxidative stress; Kampo formulation

Idiopathic pulmonary fibrosis (IPF) is a chronic progressive disorder that involves excessive extracellular matrix remodeling, resulting in severe respiratory impairment. The prevalence of IPF appears to be increasing (approximately 17/100000) and the prognosis remains poor (the mean survival time from diagnosis is 2—5 years). Inflammatory mechanisms such as those involving cytokines, reactive oxygen species and growth factors appear to be involved in the development of IPF. Accordingly, anti-inflammatory/fibrotic drugs such as pirfenidone and N-acetylcystein have been investigated, but none have succeeded in clinical trials, except for limited therapeutic effects. 

The ethical Kampo formulation Sho-seiryu-to (a Chinese/Japanese traditional medicine) is used throughout south-east Asia. It is a mixture of eight herbal components: Pinellia Tuber, Glycyrrhiza Root, Cinnamon Bark, Schisandra Fruit, Asiasarum Root, Paeony Root, Ephedra Herb and Ginger Rhizome, and in Japan has been used in the treatment of pulmonary diseases such as asthma, bronchitis and allergic diseases. Basic and clinical studies have been conducted to confirm the usefulness of Sho-seiryu-to and TJ-19 (Sho-seiryu-to extract granules for ethical use). Our recent study indicated that TJ-19 drastically attenuated oleic acid-induced lung injury, a representative animal model of acute lung injury with severe hypoxemia and pulmonary edema, at least in part through antioxidative and anti-inflammatory effects. Based on these lines of evidence, TJ-19 may also be effective against inflammatory pulmonary diseases; however, little has been reported on its effects against pulmonary fibrosis.

This study was conducted to examine whether or not TJ-19 has the potential to attenuate pulmonary fibrosis, thus identifying it as a drug candidate against the disease. Using bleomycin (BLM)-induced pulmonary fibrosis, a representative model of pulmonary fibrosis, we examined the effects of TJ-19 on body weight loss, changes in lung/body weight ratio, collagen accumulation and oxidative stress in the lungs, and historical changes induced by BLM in rats.

MATERIALS AND METHODS

Materials TJ-19 (Lot 23023392) was purchased from Tsumura & Co. (Tokyo, Japan). BLM (Lot 230110) was kindly provided by Nippon Kayaku Co., Ltd. (Tokyo, Japan). SP Histostain TM-Plus Kits were obtained from Beijing Zhongshan Goldenbridge Biotechnology Co., Ltd. (Beijing, China). Hydroxyproline standard was obtained from Sigma Chemical Co. (St. Louis, U.S.A.). The malondialdehyde (MDA) detecting kit was obtained from Nanjing Jiancheng Biochemical Institute (Beijing, China). Other reagents and solvents were of reagent grade. Deionized and distilled water was used throughout the study.

Three-Dimensional HPLC Analysis To analyze the chemical constituents of TJ-19, three-dimensional HPLC was performed as reported previously. Briefly, a granule of TJ-19 (1.0 g) was extracted with methanol (20 ml) under ultrasonication for 30 min and then centrifuged at 3000 rpm for 5 min. The supernatant was filtrated with a membrane filter (0.45 m) and then submitted for HPLC analysis (30 µl). Three independent samples were prepared and analyzed to confirm reproducibility. HPLC apparatus consisted of an Agilent 1200 Series (Agilent Technologies, Palo Alto, U.S.A.) equipped with a multiple wavelength detector (UV

* To whom correspondence should be addressed. e-mail: y-yuka@gpo.kumamoto-u.ac.jp © 2010 Pharmaceutical Society of Japan
200—400 nm). Each component was identified by comparing the peak retention times and wavelengths with those of authentic compounds using a computer system originally developed by Tsumura & Co. for the identification of the components of ethical Kampo formulation. HPLC conditions were as follows: column, ODS (TSK-GEL 80TS, 250 x 4.6 mm i.d.; Tosoh, Tokyo, Japan); eluent, (A) 0.05 M ammonium acetate (pH 3.6), (B) 100% acetonitrile. A linear gradient of 90% A and 10% B changing over 60 min to 0% A and 100% B was used (and 100% B was continued for 20 min); temperature, 40 °C; flow rate, 1.0 ml/min.

Animal Care and Handling Sprague-Dawley (S.D.) rats were obtained from the animal house section of Yanbian University Health Science Center, China. The animals were housed in stainless steel metabolite cages and observed under a natural light–dark cycle in a well-ventilated room at 23 ± 1 °C. They were fed with standard pellet food and tap water ad libitum. The study was approved by the Animal Care and Use Committee of Yanbian University Medical College, and was performed in accordance with the National Institute of Health guidelines for the care and handling of animals.

Administration of Reagents and Study Groups Thirty-six S.D. rats (male, 7—8 weeks old, 210 ± 20 g) were randomly divided into the following 4 groups (Fig. 1): sham operation group (sham group); BLM control group (BLM group); TJ-19 pre-treatment group (Pre TJ-19 group); and TJ-19 simultaneous treatment group (Simultaneous TJ-19 group). In the sham group, rats received an intratracheal instillation of 0.5 ml/kg 0.9% sterile saline via tracheotomy under diethyl ether anesthesia. In the BLM group, rats received an intratracheal instillation of a single dose of BLM (5 mg in 0.5 ml sterile saline/body weight (kg)). The sham and BLM groups received saline (oral, 5 ml/body weight (kg)) twice a day for 4 weeks after intratracheal instillation. We selected the doses of TJ-19 based on our previous report and small scale preliminary experiments. To confirm the prophylactic potentials of TJ-19 against BLM-induced pulmonary fibrosis, we employed two different administration methods, namely, Pre- and Simultaneous TJ-19 treatments. In the Pre TJ-19 group, administration of TJ-19 was started 1 week before the intratracheal instillation of BLM and then TJ-19 was given consecutively for 4 weeks. In the Simultaneous TJ-19 group, administration of TJ-19 was started just before the instillation of BLM and then given for 4 weeks. TJ-19 (oral, 1.5 g of TJ-19 in 5 ml saline/body weight (kg)) was administered twice a day to both the Pre and Simultaneous TJ-19 groups. On the 28th day, the rats were anesthetized with pentobarbital sodium (50 mg/kg) intraperitoneally. Following exsanguination, the chest cavity was opened. The left lung was used for morphological observation, while the right was divided into two parts; half was embedded in acetone for hydroxyproline measurements and the remaining half was frozen in liquid nitrogen for determination of MDA.

Histological Examination The left lung specimens were fixed in the distended state by infusion of 10% formalin into the trachea and then left for 1 week. Hematoxylin and eosin (HE) staining was performed according to conventional methods. Histological diagnosis was performed by pathologists at the department of pathology, affiliated Hospital of Yanbian University (Professor Dong-ming Piao). The assignment of study groups was blinded to the pathologists.

Measurements of Lung/Body Weight Ratio, Collagen Deposition and Lipid Peroxides The body weight of all animals was monitored every morning until sacrifice. After thoracotomy, the lungs were harvested then weighed to determine the lung/body weight ratio. The collagen content in the lung tissue was estimated by measuring hydroxyproline according to the method of Nagatani et al. The level of lipid peroxides in the tissue was determined as thiobarbituric acid-reactive substance using an MDA detecting kit.

Statistical Analysis Results were expressed as mean ± S.E.M. Multiple comparisons were performed to examine the statistical significance of the data. When uniform variance of data was identified by Bartlett’s analysis (p < 0.05), one-way analysis of variance (ANOVA) or repeated measures ANOVA was used to test for statistical differences. When significant differences (p < 0.05) were identified, the data were further analyzed by Tukey’s multiple range test for significant differences among values. If uniform variance of data was not identified, non-parametric multiple comparisons were made. After confirming significant differences (p < 0.05) using Kruskal–Wallis analysis, the differences were examined by applying Dunn’s test.

RESULTS

Three-Dimensional HPLC Analysis of TJ-19 The HPLC profile of the TJ-19 extract is shown in Fig. 2. At least 20 constituents were identified including liquiritin and glycyrrhizin (originating from Glycyrrhiza Root), paoniflorin and albiflorin (Paeony Root), schizandrin (Schisandra Fruit), cinnamic aldehyde and cinnamic acid (Cinnamon Bark) and 6-gingerol (Ginger Rhizome). This result was in accordance with our previous study using a different lot of TJ-19. On the other hand, a portion of scarce constituents, for example, procyanidin C-1, PT-H-16, which was previously identified by Amagaya et al. was not detected in the current study.

General Appearance and Changes in Body Weight Rats in the sham group were in a good state, with normal food intake and increased body weight. In contrast, on the 2nd day after BLM injection, most rats were found to have a cough, which lasted for approximately 10 day. In addition, marked polypnea, fine bibasilar inspiration crackles, listlessness, dullness, anorexia, and fluffing and withering of the fur were observed in the BLM group. Changes in body weight after BLM instillation are shown in Fig. 3. After BLM injection, all rats showed a decrease in body weight,
and significant differences were observed compared with the sham group. Rats in the Pre and Simultaneous TJ-19 groups were generally healthier than those in the BLM group. The body weight decreases in the Pre and Simultaneous TJ-19 groups were slightly reduced compared with the BLM group (Fig. 3).

**Histological Changes** In the sham group, there were visible simple columnar epithelium or cuboidal epithelium respiratory bronchioles, a few alveolar outlets in the walls of the respiratory bronchioles, spherical inflation in the terminal of the alveolar septum, and a regular structure in the walls of pulmonary alveoli (Fig. 4A). In the BLM group, there was significant thickening of alveolar walls with a disorganized alveolar structure and markedly thickened interalveolar septum. Some of the alveolar cavities became bag-shaped with broken alveolar walls, and some were atrophic with accumulated exudates, edema or inflammatory infiltration (Fig. 4B).

Abnormal changes attributed to the BLM injection were also observed; the extent and severity of alveolar and fibrosis seemed to be milder in the Pre and Simultaneous TJ-19 group than in the BLM group (Figs. 4C, D, respectively). In addition, we determined the preventative potential of TJ-19 against collagen deposition induced by BLM injection through Sirius-red staining and immunohistochemistry for Collagen III (data not shown).

**Changes in Lung/Body Weight Ratio, Collagen Deposition and Lipid Peroxides** As shown in Fig. 5, the lung/
HPLC data indicated that TJ-19 contains antioxidative components, in part, can be ascribed to antioxidative potential. The 3D-HPLC analysis revealed an increase in MDA content in the lung after BLM administration. This result suggests that the preventive effects of TJ-19 against BLM toxicity, particularly pulmonary fibrosis, and these may also affect the inhibitory effects of TJ-19 against pulmonary fibrosis. Liquiritin and Simultaneous TJ-19 groups, these parameters were lower than in the BLM group.

DISCUSSION

In this study, we demonstrated that multiple administration of TJ-19 suppressed the body weight loss, increases in lung/body weight ratio, and hydroxyproline and MDA contents induced by intratracheal injection of BLM when administered 1 week before or simultaneously with BLM injection. To examine and confirm the prophylactic properties of TJ-19, we designed two TJ-19 treatment groups: the Pre TJ-19 and Simultaneous TJ-19 groups. There were no significant differences in any of the parameters between the two groups. These results indicate that TJ-19 has prophylactic potential against BLM toxicity, particularly pulmonary fibrosis, and suggest that TJ-19 is a candidate drug for the prevention of IPF.

In our previous study, we demonstrated that TJ-19 has antioxidative properties that appeared to result in attenuation of the acute lung injury induced by oleic acid. Reactive oxygen species (ROS) play a crucial role in the development of pulmonary fibrosis induced by BLM. In this study, we observed an increase in MDA content in the lung after BLM injection; however, this increase was attenuated by TJ-19 administration. This result suggests that the preventive effects of TJ-19 on BLM-induced pulmonary fibrosis, at least in part, can be ascribed to antioxidative potential. The 3D-HPLC data indicated that TJ-19 contains anti-oxidative constituents including catechin, 6-gingerol, and glycyrrhizin, further supporting the above suggestion. However, in addition to ROS, other inflammatory mediators and mechanisms are involved in the development of BLM-induced pulmonary fibrosis, and these may also affect the inhibitory effects of TJ-19 against BLM-induced pulmonary fibrosis. Liquiritin derivatives, paoniflorin and gomisins, were also detected in TJ-19 by 3D-HPLC and are known to have anti-inflammatory effects including inhibition of cytokine release and an anti-apoptotic effect.

In addition, a recent study indicated that glycyrrhizin, another constituent of TJ-19, suppresses cytokine production and nuclear factor-kappa B activity, important factors in the development of BLM-induced pulmonary fibrosis. These constituents may also act additively or synergistically in the inhibitory potential of TJ-19 against BLM-induced pulmonary fibrosis. Future studies to examine the yield of the anti-oxidative components in TJ-19 and the effect of each component on this pulmonary fibrosis model will be needed.

BLM is known to be a representative inducer of pulmonary fibrosis and is used in experimental models of IPF. BLM has also been clinically used as a chemotherapeutic agent for the treatment of malignant conditions such as squamous cell carcinomas, lymphomas and germ cell tumors. However, its clinical use is limited by the risk of severe pulmonary toxicity, and there is as yet no established treatment for the prevention of this adverse reaction. The results of this study suggest, however, that TJ-19 has potential as a prophylactic agent for the prevention of pulmonary toxicity in patients administered BLM.

Although the results of this study suggest the prophylactic potential of TJ-19 against IPF and the adverse events of BLM, for clinical use it remains important to clarify whether or not TJ-19 has curative as well as prophylactic effects against developing pulmonary fibrosis. In addition, the precise mechanisms and inhibitory constituents of TJ-19 against BLM-induced pulmonary fibrosis also need to be determined. Further study is therefore warranted.

In summary, we demonstrated the beneficial effects of TJ-19 in the prevention of pulmonary fibrosis induced by BLM in rats. We suggest that this traditional Kampo formulation is an attractive candidate and source of prophylaxis against IPF and clinical pulmonary toxicity induced by BLM.

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REFERENCES AND NOTES

1) Present address: Research Division of Pharmacology, China Pharmaceutical University; Nanjing, Jiangsu Prov. 210009, China.