Journal of Ethnopharmacology 154 (2014) 687–695

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Research Paper

Anti-tumor effect of Shu-gan-Liang-Xue decoction in breast cancer is related to the inhibition of aromatase and steroid sulfatase expression

Ning Zhou [1], Shu-Yan Han [2], Fei Zhou [2], Ping-ping Li *

Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Department of Integration of Chinese and Western Medicine, Peking University Cancer Hospital & Institute, Beijing 100142, China

ARTICLE INFO

Article history:
Received 26 November 2013
Received in revised form
24 April 2014
Accepted 27 April 2014
Available online 6 May 2014

Keywords:
Breast cancer
Estrogen
Traditional Chinese medicine
Aromatase
Steroid sulfatase

ABSTRACT

Ethnopharmacological relevance: Shu-Gan-Liang-Xue Decoction (SGLXD), a traditional Chinese herbal formula used to ameliorate the hot flushes in breast cancer patients, was reported to have anti-tumor effect on breast cancer. Estrogen plays a critical role in the genesis and evolution of breast cancer. Aromatase and steroid sulfatase (STS) are key estrogen synthesis enzymes that predominantly contribute to the high local hormone concentrations. The present study was to evaluate the anti-tumor effect of SGLXD on estrogen receptor (ER) positive breast cancer cell line ZR-75-1, and to investigate its underlying mechanisms both *in vitro* and *in vivo*.

Materials and methods: The anti-tumor activity of SGLXD *in vitro* was investigated using the MTT assay. The *in vivo* anti-tumor effect of SGLXD was evaluated in non-ovariectomized and ovariectomized athymic nude mice. The effect of SGLXD on enzymatic activity of aromatase and STS was examined using the dual-luciferase reporter (DLR) based on bioluminescent measurements. Aromatase and STS protein level were assessed using Western blot assay.

Results: SGLXD showed dose-dependent inhibitory effect on the proliferation of ZR-75-1 cells with IC_{50} value of 3.40 mg/mL. It also suppressed the stimulating effect on cell proliferation of testosterone and estrogen sulfates (E_1S). Oral administration of 6 g/kg of SGLXD for 25 days resulted in a reduction in tumor volume in non-ovariectomized and ovariectomized nude mice. The bioluminescent measurements confirmed that SGLXD has a dual-inhibitory effect on the activity of aromatase and STS. Western blot assay demonstrated that the treatment of SGLXD resulted in a decrease in aromatase and STS protein levels both *in vitro* and *in vivo*.

Conclusion: Our results suggested that SGLXD showed anti-tumor effect on breast cancer cells both *in vitro* and *in vivo*. The anti-tumor activity of SGLXD is related to inhibition of aromatase and STS via decreasing their expression. SGLXD may be considered as a novel treatment for ER positive breast cancer.

1. Introduction

Breast cancer is the most common malignancy among women around the world. Approximately 60–70% of breast tumors express estrogen receptors (ER) (Chen, 1998), meaning that the tumor growth could be stimulated by estrogen. Estrogens, particularly 17β-estradiol (E_2), the most biologically active estrogen of this class of hormones, play a critical role in the development of breast cancer. The manipulation of estrogen synthesis and function has become the main endocrine therapy method for ER-positive breast cancer (Altundag and Ibrahim, 2006).

Estrogens can be locally produced *de novo* by estrogen synthesis enzymes in breast cancer tissues to promote tumor growth (Pasqualini et al., 1996). The estrogen level in tumor tissue is significantly higher than the concentration in circulation (Shibuya et al., 2008). Aromatase and steroid sulfatase (STS) are key estrogen synthesis enzymes that predominantly contribute to the high local hormone concentrations. Inhibiting enzymatic synthesis of estrogens is therefore an effective endocrine treatment method for postmenopausal women with ER-positive breast cancer (Altundag and Ibrahim, 2006). Aromatase is responsible for the conversion of

Abbreviations: SGLXD, Shu-Gan-Liang-Xue Decoction; STS, steroid sulfatase; ER, estrogen receptor; DLR, dual-luciferase reporter; E_2, 17β-estradiol; A_4, androstenedione; E_1, estrone; AIs, aromatase inhibitors; E_1S, estrone sulfates; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; FBS, fetal bovine serum; ERE, estrogen response element

* Corresponding author. Tel./fax: +86 10 88196069.
E-mail addresses: zndf1986@163.com (N. Zhou), shuyanhan@126.com (S.-Y. Han), feieryanjiu@163.com (F. Zhou), lppma123@126.com (P.-p. Li).
[1] Tel.: +86 10 88196841.
[2] Tel.: +86 10 88196706.

http://dx.doi.org/10.1016/j.jep.2014.04.045
0378-8741/

testosterone and androstenedione (A_4) to E_2 and estrone (E_1) respectively (Speirs et al., 1999). Aromatase inhibitors (AIs), such as anastrozole and letrozole, have been widely used for breast cancer treatment in clinical practice. Despite the success of AIs, numerous breast cancer patients still progress after AI therapy (Taylor et al., 1982). STS is another key enzyme for estrogen production through the hydrolysis of estrone sulfates (E_1S) to E_1 (Reed et al., 2005). A high level of STS mRNA expression is associated with breast tumors progression (Miyoshi et al., 2003) and STS is thus considered to be a promising target for the treatment of ER-positive breast cancer (Duncan et al., 1993; Geisler et al., 2011). The inhibition of STS with STX64 in breast tumors has obtained encouraging results in preclinical experiments and clinical trials (Howarth et al., 1994; Woo et al., 1998). Previous studies have demonstrated significantly declined estrogen levels through simultaneous inhibition of aromatase and STS activity. Therefore, dual inhibition of aromatase and STS may be a potentially effective therapy for ER-positive breast cancer (Howarth et al., 1994; Wang et al., 2009). However, no drugs capable of inhibiting both aromatase and STS have been approved for clinical application to data.

The anti-tumor potential of Chinese herbal medicines on breast cancer has been evaluated in the past few decades. Some herbs were shown to suppress the growth of estrogen-dependent breast tumor cells by reducing the enzymatic synthesis of estrogen. Several compounds isolated from herbs are known to be potent aromatase inhibitors, such as hesperetin, naringenin and apigenin (Wang et al., 1994; Jeong et al., 1999; Joshi et al., 1999; Paoletta et al., 2008; van Meeuwen et al., 2008; Li et al., 2011). However, the majority of these studies only evaluated the anti-tumor effect of the herbs *in vitro*, and the regulatory effect of these particular herbs on estrogen biosynthesis and on key enzyme expression and activity *in vivo* remain unknown.

Shu-Gan-Liang-Xue Decoction (SGLXD) is a cipher prescription that has been used for the treatment of hot flashes in breast cancer patients (Li, 2004) and its therapeutic effects have been confirmed in a randomized, double-blind, placebo-controlled trial (Sun et al., 2009). A previous study has also shown that SGLXD inhibits the growth of the breast cancer cell lines MCF-7 and T47D (Zhang and Li, 2010; Fu and Li, 2011). However, the effects observed *in vitro* do not necessarily agree with the *in vivo* results. The anti-tumor effect of SGLXD and the mechanisms of action *in vivo* thus remain unclear. ZR-75-1, a human ER-positive breast cancer cell line with a natural expression of aromatase and STS at high levels (Theriault and Labrie, 1991; Ishida et al., 2007), has been extensively used in breast cancer research. The aim of the present study was to examine the inhibitory effect of SGLXD on the growth of ZR-75-1 cells *in vitro* and *in vivo* and to investigate the underlying mechanisms.

2. Materials and methods

2.1. Herbal materials and SGLXD extract

The component herbs of SGLXD used in this study are as follows (with voucher numbers): Cortex Moutan (dried root bark of *Paeonia suffruticosa Andrews*, in *Paeoniaceae*), Fructus Schisandrae (dried fruit of *Schisandra chinensis (Turcz.) Baill*, in *Schisandraceae*), Radix Paeoniae Alba (dried root of *Paeonia lactiflora Pall*, in *Paeoniaceae*), Radix Cynanchi Atrati (dried root of *Cynanchum atratum Bunge*, in *Asclepiadaceae*), Radix Bupleuri (dried root of *Bupleurum chinense DC*, in *Apiaceae*), and Radix Curcumae (dried root tuber of *Curcuma aromatica Salisb*, in *Zingiberaceae*), at a ratio of 3:3:3:3:2:2. The herbs of SGLXD were purchased from Bai-Ta-Si Pharmacy (Beijing, China). All of the herbs are regionally famous drugs and were authenticated by the herbalists of Bai-Ta-Si Pharmacy. The authenticated voucher specimens are available at our department as voucher numbers No. 20110720-CM for Cortex Moutan, No. 20110720-FS for Fructus Schisandrae, No. 20110720-RPA for Radix Paeoniae Alba, No. 20110720-RCA for Radix Cynanchi Atrati, No. 20110720-RB for Radix Bupleuri, and No. 20110720-RC for Radix Curcumae.

SGLXD was extracted as previously described (Zhang and Li, 2010), concentrated, and converted into a spray-dried powder at a yielding rate of 16.7%. The powder extract was dissolved in distilled water and filtered through a 0.22-μm membrane (Millipore, Billerica, MA, USA), prior to experimentation. The drug concentrations described in our experiment indicated the concentrations of the SGLXD powder extract.

2.2. Chemicals and antibodies

Anastrozole (Arimidex) was purchased from AstraZeneca Pharmaceuticals. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), 17β-estradiol (E_2), testosterone, estrone sulfate (E_1S), and STX64 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit polyclonal antibodies to STS and aromatase were purchased from Abcam (Cambridge, MA, USA). The β-actin mouse monoclonal antibody was purchased from Sinoble Biotech Labs (Beijing, China). The peroxidase-conjugated AffiniPure goat anti-rabbit IgG and goat anti-mouse IgG were purchased from the ZSGB-Bio Company (Beijing, China).

2.3. Cell culture

The ZR-75-1 human breast cancer cell line was purchased from the cell center of the Peking Union Medical College. The cells were cultured in RPMI-1640 supplemented with 15% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) and incubated at 37 °C in a humidified chamber with 5% CO_2.

2.4. Cell proliferation assay

The ZR-75-1 cells were seeded in 96-well plates at a density of 2×10^5 cells/ml and allowed to attach overnight. The cells were treated with indicated concentrations of SGLXD for 24 h, and the solvent of SGLXD served as the vehicle control. The cells were incubated with MTT (at a final concentration of 0.5 mg/ml) at 37 °C for 4 h, and the precipitate was dissolved in 100 μl of dimethyl sulfoxide (DMSO). After shaking for 10 min, the absorbance at a wavelength of 570 nm was detected using a microplate reader (Model 680, Bio-Rad, Hercules, CA, USA). Each treatment was performed in quadruplicate. The values were compared with the values of the control group, which were set to 100%. Based on the results of our preliminary experiment, 1.70, 3.40, and 5.10 mg/ml SGLXD (equal to crude drug 10 mg/ml, 20 mg/ml, and 30 mg/ml) were selected for application in the subsequent experiments.

Aromatase is responsible for the conversion of testosterone to E_2, and the hydrolysis of E_1S to E_1 (precursor of E_2) is catalyzed by STS. The product of aromatase and STS stimulates the growth of ER-positive breast cancer cells. Testosterone (enzyme substrate of aromatase) and E_1S (enzyme substrate of STS) were supplemented to evaluate the catalytic activity of aromatase and STS. After growing for 24 h, the ZR-75-1 cells were washed with PBS and the medium was replaced with fresh growth medium containing 0.2 mM testosterone or 0.2 mM E_1S. The cells were then treated with the aromatase inhibitor anastrozole (100 μmol/l), the STS inhibitor STX64 (20 μmol/l), or SGLXD for 24 h. Cell proliferation was evaluated by performing a MTT assay, as described.