Department of Anatomy[1], School of Preclinical Medicine, Beijing University of Chinese Medicine; Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education)[2], Department of Integrated Traditional Chinese and Western Medicine, Peking University School of Oncology, Beijing Cancer Hospital & Institute; Department of Integrated Traditional Chinese and Western Medicine[3], Peking University School of Basic Medical Science, Beijing, China

Assessment of shikonin for potential estrogenic activity by dual-luciferase reporter based bioluminescent measurements *in vitro*

Yi Zhang [1,3], Ping-Ping Li [2]

Received July 15, 2010, accepted August 13, 2010

Ping-Ping Li, Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Department of Integrated Traditional Chinese and Western Medicine, Peking University School of Oncology, Beijing Cancer Hospital & Institute, Beijing 100142, China
lppma123@yahoo.com.cn

Pharmazie 66: 141–143 (2011) *doi: 10.1691/ph.2011.0714*

Shikonin, an active component of *Lithospermum erythrorhizon* Sieb. et Zucc., shows multiple pharmacological properties. However, the estrogenic activity of shikonin is remaining unclear. We assessed the potential estrogenic activity of shikonin with dual-luciferase reporter assay and bioluminescent measurements, by using transient cotransfection with estrogen dependent plasmid pERE-TK-Luc and internal control plasmid pRL-TK in MCF-7 cells. Estrogenic activity of shikonin, even at high concentration did not alter significantly compared to negative control ($p > 0.05$) and were significantly lower than those with E_2 ($p < 0.01$). Concluding, shikonin demonstrates no estrogenic activity *in vitro*.

1. Introduction

The naphthoquinone pigment, shikonin, is the major active component isolated from the Chinese herbal drug, Zicao (*Lithospermum erythrorhizon* Sieb. et Zucc.) that has been used for thousands of years. Like its genitor plant, shikonin possesses multiple pharmacological properties, e.g., anti-inflammatory (Chen et al. 2002; Staniforth et al. 2004), anti-arthritis (Dai et al. 2009) and anti-tumor (Singh et al. 2003; Zhang et al. 2009) activity.

Chinese medicinal herbs have been used as natural medicines to heal many diseases. However, many herbs contain estrogenic substances that have a structural and functional similarity to estrogen (Ruggiero and Likis 2002). Estrogens exert their biological effects in many physiological processes through changing the expression profile of genes involved in cell proliferation and differentiation (Hall et al. 2001). The excess of estrogen can cause breast, endometrial, ovarian, and prostate cancer due to unselective estrogenic action (Henderson et al. 1988).

It is noteworthy that no reports of shikonin's potential estrogenic activity on the cytology have been found. Therefore, as part of an extensive program of safety evaluation researches, the potential estrogenic activity of shikonin was assessed in present research.

2. Investigations, results and discussion

Traditional Chinese medicines (TCMs) have been used in China for thousands of years, but the relationship and mechanism between safety and efficiency are still unclear owing to the complexity of the constituents. The bioactivity of phytocompounds present in herbs has attracted more attention, especially the estrogenic activity, due to their possible involvement in the modulation of various dysfunctions and diseases (Ruggiero and Likis 2002).

It was previously reported that *L. erythrorhizon* Sieb. et Zucc. has antiproliferative activity on MCF-7 cells (Wang and Li 2003). Moreover, the aqueous extract of *L. erythrorhizon* does not manifest estrogenic activity at the crude herb concentration of 1 mg/ml *in vitro* (Zhang and Li 2009). According to a preliminary cell proliferation assay (Zhang et al. 2009), shikonin displays antiproliferative effects on MCF-7 cells. The value of IC_{50} is about 10.30 μM. The doses of 2.5, 5, 10 and 20 μM were used consequently in the present estrogenic activity study.

Estrogenic activity of shikonin was compared with that of E_2 at a concentration of 0.1 μM (positive control) and of MCF-7 cells treated without any chemical (negative control). The luciferase activity inductions with different concentrations of shikonin were significantly lower than those with E_2 (0.1 μM) alone ($p < 0.01$) and were not statistically significant difference compared to negative control ($p > 0.05$; Fig. 1). Briefly, shikonin did not demonstrate estrogenic activity, even at high concentrations.

Estrogenic activity has been assessed intensively among a great deal of substances including phytocompounds, medicines, pesticides and industrial chemicals using various techniques (De Naeyer et al. 2005; Gutendorf and Westendorf 2001; Sookvanichsilp et al. 2008). According to well-elucidated mechanisms of action (Tsai and O'Malley 1994), *in vitro* methods use more definitive end points and are more sensitive than *in vivo* methods. The method system based on the binding of estrogen-receptor complex to estrogen response elements (EREs) is the simplest among the *in vitro* methods. For carrying the human estrogen receptor gene, MCF-7 cells are very suitable for large-scale screening and sensitive analysis of estrogenic substances (Cherdshewasart and Sriwatcharakul 2008). Such knowledge is

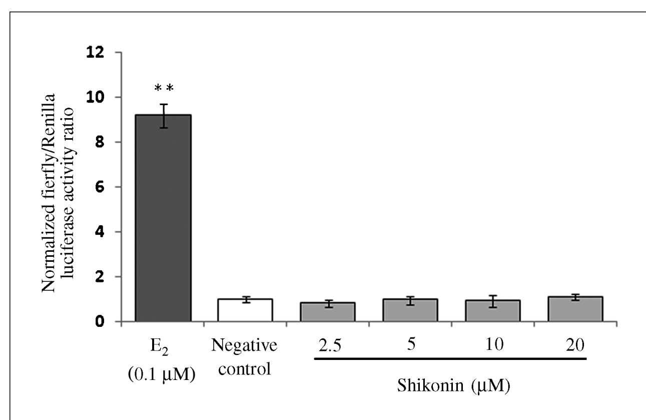

Fig. 1: Estrogenic activity of shikonin by DLR assay. One day after transfection, cells were incubated in fresh culture medium containing E_2 (0.1 μM) and various concentrations of shikonin (2.5, 5, 10 and 20 μM) for 24 h, respectively. Firefly luciferase activity was normalized to that of *Renilla*, and results were expressed as mean fold induction over negative control which has been normalized to 1.0. **: $p < 0.01$

a critical prerequisite for the development of safe and efficacious traditional medicine herbs.

Our previous research showed that dual-luciferase reporter (DLR) assay system captured a dose response to estrogen in MCF-7 cells (Fig. 2) (Zhang and Li 2009), demonstrating the sensitivity of DLR assay system and bioluminescence quantification *in vitro*. We believe that this method is readily applicable to the area of medicinal science.

The limitation of *in vitro* methods is also that they do not account for differences in uptake of the phytocompound, bioaccumulation or interactions involving the induction of binding proteins such as sex hormone binding globulins that may modulate the uptake and metabolism of sex steroids or steroid mimics. Future *in vivo* studies are also required to assess the estrogenic activity of shikonin.

Concluding, shikonin does not manifest estrogenic activity *in vitro* but is a promising natural compound which holds promise for the future design of more potent therapeutics.

3. Experimental

3.1. Chemicals and reagents

Shikonin ($C_{16}H_{16}O_5$; molecular weight: 288.3) was purchased from Calbiochem (Merck, Darmstadt, Germany). 17β-Estradiol (E_2) was purchased

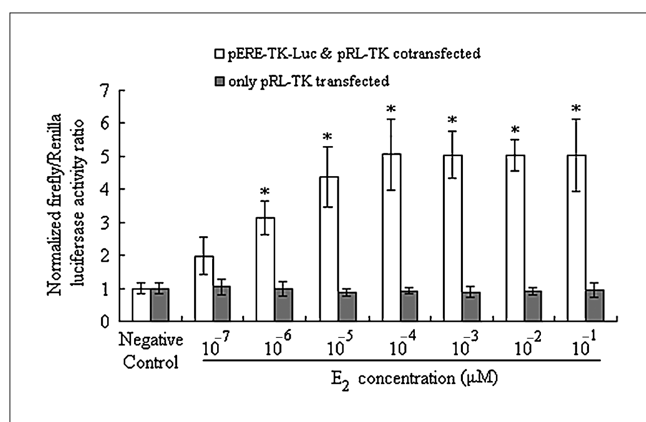

Fig. 2: Effects of estrogen dose response by the DLR system when pRL-TK transfected with the present and absent of pERE-TK-Luc respectively (Zhang and Li 2009). One day after transfection, fresh mediums containing various doses of E_2 (10^{-7} to 10^{-1} μM) were added, DLR assay was performed after incubation for 24 h. For cells cotransfected with pERE-TK-Luc and pRL-TK, treatment with increasing dose of E_2 results in a statistically significant increase in luciferase activity induction compared with negative control ($p < 0.05$). For cells only transfected with internal pRL-TK, induction of luciferase activity is not altered compared to negative control statistically ($p > 0.05$)

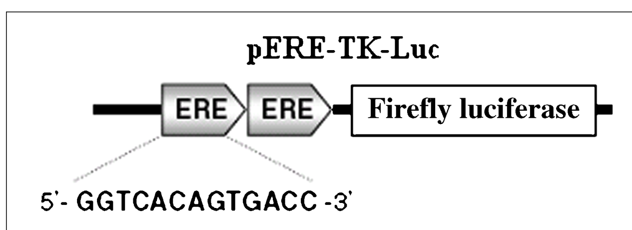

Fig. 3: Concise structure of two tandem EREs in pERE-TK-Luc

from Sigma-Aldrich (St. Louis, MO, USA). All other chemical reagents used in present research were of analytical grade, obtained from Beijing Chemical Factory (Beijing, China).

3.2. Cell line and culture

Human breast carcinoma cell line MCF-7 (ATCC No.HTB-22) was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and was grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS, Gibco BRL, Grand Island, NY, USA), 1 mM nonessential amino acids, 0.1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2.

3.3. Plasmids

Plasmid pERE-TK-Luc (Fig. 3) contains two tandem copies of the palindromic estrogen response element (ERE) sequence, directing expression of the firefly (*Photinus pyralis*) luciferase coding sequence in response to transcriptional activation by estrogen-bound estrogen receptor. Plasmid pRL-TK contains the minimal TK promoter driving expression of a humanized *Renilla* (*Renilla reniformis,* also known as sea pansy) luciferase and was used as the internal control of pERE-TK-Luc.

3.4. Transient transfection and cell treatments

Two days before transient transfection, cells were transferred to phenol red-free DMEM (Hyclone, Logan, UT, USA) supplemented with 10% charcoal-stripped FBS (Biological Industries, Kibbutz Beit Haemek, Israel). One day before transient transfection, cells were seeded in 24-well plates at a density of 2.0×10^5 cells/ml. Cells were cotransfected with 0.80 μg of pERE-TK-Luc and 0.04 μg of pRL-TK using transfection reagent Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) for each well according to the manufacturer's protocol. Twenty four hours after transfection, cells were incubated in fresh culture medium containing E_2 (0.1 μM) and various concentrations of shikonin (2.5, 5, 10 and 20 μM) for 24 h, respectively.

3.5. Dual-luciferase reporter (DLR) assay and bioluminescent measurements

The firefly and *Renilla* luciferase activity was measured in the same well sequentially from a single sample, using DLR assay kit (Promega, Madison, WI, USA) according to the protocol. The bioluminescence was measured with a microplate-reading luminometer LMax II (Molecular Devices, Sunnyvale, CA, USA) according to the manufacturer's instruction. The bioluminescent measurement data were recorded with software SoftMax, version ProV5 (Molecular Devices, Sunnyvale, CA, USA). All the experiments were performed in triplicates and three independent experiments.

3.6. Statistical analysis

All results were expressed as mean ± standard deviation (SD) and were analyzed by one-way ANOVA, with the Dunnett's *t*-test. All data were processed with statistical analysis software SPSS, version 16.0 (SPSS Inc., Chicago, IL, USA). For this research, the statistical significant difference was set at *: $p < 0.05$ and **: $p < 0.01$.

Acknowledgement: The Key Program Foundation of Beijing Administration of TCMs (2004-IV15), China, supported this research.

References

Chen X, Yang L, Oppenheim JJ Howard MZ (2002) Cellular pharmacology studies of shikonin derivatives. Phytother Res 16: 199–209.

Cherdshewasart W, Sriwatcharakul S (2008) Metabolic activation promotes estrogenic activity of the phytoestrogen-rich plant. Maturitas 59: 128–136.

Dai Q, Fang J, Zhang FS (2009) Dual role of shikonin in early and late stages of collagen type II arthritis. Mol Biol Rep 36: 1597–1604.
De Naeyer A, Pocock V, Milligan S, De Keúkeleive (2005) Estrogenic activity of a polyphenolic extract of the leaves of Epimedium brevicornum. Fitoterapia 76: 35–40.
Gutendorf B,Westendorf J (2001) Comparison of an array of in vitro assays for the assessment of the estrogenic potential of natural and synthetic estrogens, phytoestrogens and xenoestrogens. Toxicology 166: 79–89.
Hall JM, Couse JF, Korach KS (2001) The multifaceted mechanisms of estradiol and estrogen receptor signaling. J Biol Chem 276: 36869–36872.
Henderson BE, Ross R, Bernstein L (1988) Estrogens as a cause of human cancer: the Richard and Hinda Rosenthal Foundation award lecture. Cancer Res 48: 246–253.
Ruggiero RJ, Likis FE (2002) Estrogen: physiology, pharmacology, and formulations for replacement therapy. J Midwifery Womens Health 47: 130–138.
Singh F, Gao D, Lebwohl MG, Uei H (2003) Shikonin modulates cell proliferation by inhibiting epidermal growth factor receptor signaling in human epidermoid carcinoma cells. Cancer Lett 200: 115–121.
Sookvanichsilp N, Soonthornchareonnon N, Boonleang C (2008) Estrogenic activity of the dichloromethane extract from Pueraria mirifica. Fitoterapia 79: 509–514.
Staniforth V, Wang SY, Shyur LF, Rang NS (2004) Shikonins, phytocompounds from *Lithospermum erythrorhizon*, inhibit the transcriptional activation of human tumor necrosis factor alpha promoter in vivo. J Biol Chem 279: 5877–5885.
Tsai MJ, O'Malley B (1994) Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem 63: 451–486.
Wang W, Li PP (2003) The effects of herb lithospermum extract on MCF-7 cell and estrogen and progestogen levels in mice. Zhongguo Zhong Yao Za Zhi 28: 1062–1067.
Zhang Y, Qian RQ, Li PP (2009) Shikonin, an ingredient of *Lithospermum erythrorhizon*, down-regulates the expression of steroid sulfatase genes in breast cancer cells. Cancer Lett 284: 47–54.
Zhang Y, Li PP (2009) Evaluation of estrogenic potential of Shu-Gan-Liang-Xue Decoction by dual-luciferase reporter based bioluminescent measurements *in vitro*. J Ethnopharmacol 126: 345–349.
Zhang Y, Li PP (2010) Shu-Gan-Liang-Xue Decoction, a Chinese herbal formula, down-regulates the expression of steroid sulfatase genes in human breast carcinoma MCF-7 cells. J Ethnopharmacol 127: 620–624.