PHYTOTHERAPY RESEARCH
Phytother. Res. **15**, 487–492 (2001)
DOI: 10.1002/ptr.767

A Reporter Gene Assay for the Detection of Phytoestrogens in Traditional Chinese Medicine

D. M. Miller-Martini,[1] R. Y. K. Chan,[2] N. Y. Ip,[1,2] S. J. Sheu,[3] and Y. H. Wong[1,2]*

[1]The Biotechnology Research Institute, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China
[2]Department of Biology, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China
[3]Department of Chemistry, National Taiwan Normal University, 88, Sec. 4, Tingchow Road, Taipei, Taiwan

Bupleurum & Peony Formula (*Jia Wei Xiao Yao San*) is a herbal formula which possesses a clinical history for the treatment of menopausal syndrome and menstrual irregularity. The present investigation reports the ability to monitor the formula's phytoestrogen content that will allow for the implementation of a standardization protocol that is based on a quantifiable biological response. Utilizing an oestrogen-sensitive chimeric receptor/reporter gene element which has been stably transfected into HeLa cells, the botanical formula was shown to induce the expression of the reporter gene, luciferase, in a dose dependent manner. Pretreatment of the HeLa cells with the botanical formula produced a 5-fold increase in bioluminescence compared with the control. Additionally, our studies showed that the response of the cells, when challenged by the botanical formula, was oestrogen specific. Pretreatment of the cells with tamoxifen effectively blocked the activation of the chimeric oestrogen receptor by the botanical formula. The cell line provides a sensitive assay that can easily detect the presence of phytoestrogens in complex botanical formulas. Copyright © 2001 John Wiley & Sons, Ltd.

Keywords: Bupleurum & Peony Formula; oestrogen receptor; menopausal syndrome; phytoestrogen; reporter gene; traditional Chinese medicine (TCM).

INTRODUCTION

Many botanicals contain phytoestrogens that are steroid-like plant compounds which may mimic or act as precursors to sex hormones. These compounds represent a diverse body of substances capable of eliciting agonist or antagonist responses possibly via a mechanism of action comparable to that of oestrogen. Thus, phytoestrogens are defined functionally and include known compounds that belong to the structural groups of isoflavones, flavonoids, lignans, phytosterols and coumestans, some of which have been previously shown to possess potent antitumour properties (Strom *et al.*, 1999). These plant-derived compounds can be identified by their ability to bind to the oestrogen receptor and to induce or attenuate a response. Endogenous oestrogen possesses a number of important biological functions that are involved in the growth, development and homeostasis of a variety of tissues (Ciocca and Roig, 1995). It is believed that many of the botanicals used for the treatment of menopausal syndrome mediate their therapeutic effects through their endogenous phytoestrogens to offset the hormonal imbalances normally associated with menopause.

Bupleurum & Peony Formula is a traditional Chinese medicine (TCM) commonly used in the treatment of menopausal syndrome, menstrual irregularity, leukorrhea, chronic endometritis, chronic hepatitis, early stage of cirrhosis, leukoderma, toxic goitre and emotional fluctuations. The principle herbs of this formula are Peony (*Paeoniae radix*), Bupleurum (*Bupleuri radix*) and Tang-kuei (*Angelicae radix*). Other components of the formula include Gardenia (*Gardeniae fructus*), Moutan (*Moutan radicis cortex*), Atractylodes (*Atractylodis rhizoma*), Hoelen (*Poria*), Ginger (*Zingiberis siccatum rhizoma*) and Licorice (*Glycyrrhizae radix*). Among these ingredients, the phytoestrogen β-sitosterol (Rosenblum *et al.*, 1993) is known to be present in Tang-kuei, Gardenia and Moutan, while Hoelen contains ergosterol (Mayr *et al.*, 1992). Some of the therapeutic actions of Bupleurum & Peony Formula can thus be ascribed to the presence of phytoestrogens. Detection of phytoestrogen content may provide a means to assess the biological efficacy of various TCMs used in the treatment of menopausal syndrome.

Over the past decade, numerous pharmaceutical companies have developed recombinant receptor/reporter gene assays to identify receptor-specific ligands with potential therapeutic applications. Recombinant receptor/reporter gene bioassays can be categorized into three broad classes: (i) endogenous promoter-regulated reporter genes; (ii) response element-regulated reporter genes; (iii) chimeric receptor/response element-regulated reporter genes. A chimeric receptor/reporter gene bioassay is available for the detection of oestrogens (Connor *et al.*, 1995). The system utilizes two different cDNA components to detect responses of cells to oestrogenic substances. The Gal4-HEGO chimeric receptor consists of the ligand binding domain of the oestrogen receptor linked to the DNA binding domain of the yeast transcription factor, Ga14. The second component is the Gal4-regulated luciferase reporter gene that consists

* Correspondence to: Dr Y. H. Wong, Department of Biology, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China.
E-mail: boyung@ust.hk
Contract/grant sponsor: Hong Kong Industry Department; Contract/grant number: Industrial Support Fund AF/178/97
Contract/grant sponsor: Sun-Ten Pharmaceutical Co. Ltd, Taiwan.

Received 15 November 1999
Accepted 6 March 2000

of the firefly luciferase cDNA regulated by the rabbit β-globin basal promoter and five tandem consensus Gal4 response elements. The use of tandem response elements has been previously found to improve reporter gene inducibility (Ponglikitmongkol *et al.*, 1990). Expression of the reporter gene is dependent upon the ligand-dependent activation of the chimeric receptor. These two constructs have been used in mammalian expression vector to transiently transfect MCF-7 cells and have been stably transfected into HeLa cells. Here, we report the use of the stably transfected HeLa cell bioassay system to assess the potential presence of phytoestrogens in a Bupleurum & Peony Formula.

MATERIALS AND METHODS

Preparation of stock solutions of Bupleurum & Peony Formula. Bupleurum & Peony Formula used in the present study was donated by the Sun-Ten Pharmaceutical Co. Limited, Taiwan. 4% (w/v) stock solutions were prepared for several production lots of the Bupleurum & Peony Formula. These stock solutions were prepared by adding 4 g of a specified lot to 100 mL of deionized water followed by the incubation of the resultant suspension at 37 °C for 3 h. The suspensions were then centrifuged at 3000 rpm for 15 min. The resultant supernatants were collected and sterilized using 0.2 μm syringe filters before assaying.

Chimeric constructs and host cell line. A HeLa cell line that has been stably transfected with oestrogenic responsive, chimeric receptor/reporter gene elements (Connor *et al.*, 1995) was kindly provided by Dr Hinrich Gronemeyer (Institut de Genetique et de Biologie Moleculaire et Cellulaire, de Strasbourg, France). The Gal4-HEGO chimeric receptor consists of the ligand binding domain of the oestrogen receptor linked to the DNA binding domain of the yeast transcription factor, Gal4. The reporter element is composed of the luciferase gene under the regulation of the rabbit β-globin basal promoter and a five-tandem consensus of the Gal4 DNA response element. The tandem response elements were used to improve reporter gene inducibility. Bioluminescence of the system is contingent upon the ligand-dependent activation of the chimeric receptor by oestrogenic compounds.

Pretreatment of the chimeric/reporter gene HeLa cell line with stock solutions. HeLa cells stably transfected with oestrogenic responsive chimeric receptor/reporter gene elements were cultured in DMEM/10% FBS at 37 °C in a 5% CO_2 atmosphere. The cell density of the culture was maintained below 70%. Prior to the assay, the cells were deprived of oestrogen for at least 5 days by culturing the cells in DMEM (without phenol red) supplemented with 10% steroid-stripped dextran-coated charcoal serum. The assay was initiated by first seeding the culture into a 96-well microtitre plate at a density of 10 000 cells/well. The various wells containing the cultured cells were then incubated at 37 °C in 5% CO_2 for 24 h in the presence or absence of 30 μM tamoxifen, in addition to either: (i) buffer solution, (ii) various concentrations (ranging from 1×10^{-8} to 1×10^{-14} M) of 17-β-oestradiol, or (iii) various dilutions (ranging from 10- to 100 000-fold) of a 4% stock solution of the formula.

Luciferase reporter gene assay. Bioluminescence in the pretreated HeLa cells was assayed using a commercially available luciferase reporter gene assay kit. All assays were performed according to a standard protocol that had been supplied by the manufacturer (Promega) of the kit. Briefly, pretreated cells were washed twice with PBS at room temperature before adding 20 μL of lysis buffer to each well. Cells were then lysed by one round of freeze thawing by first placing the plate in a −80 °C freezer for 30 min before thawing the frozen plate at room temperature. The cell lysates were then transferred to microcentrifuge tubes where the cellular debris was removed by spinning the tubes in a microcentrifuge for 30 s. The supernatants were then transferred into microcentrifuge tubes and were used immediately to initiate the reactions. All reactions were performed in a 96-well microtitre plate format. A total of 50 μL of each of the various supernatants were transferred into separate wells followed by the automated injection into each of the wells of 100 μL of luciferase assay reagent. Light emission at 562 nm was measured for 5 s immediately after the start of the reaction using a Perkin-Elmer Applied Biosystems, TR717 Microplate Luminometer equipped with an automatic injector system. Total light emission was calculated using an integrated light analysis methodology and was plotted as Arbitrary Light Units vs either Concentration of 17-β-oestradiol or Total Dilution of the test compound. Each assay was performed in triplicate.

RESULTS

Determination of the responsiveness of the chimeric receptor/ reporter gene in stably transfected HeLa cells.

In order to determine the sensitivity of the assay system to oestrogenic compounds, the prototypic agonist (17-β-oestradiol) for the oestrogen receptor was assayed for its ability to induce expression of the luciferase reporter. Figure 1 shows the dose-dependent induction of luciferase activity when challenged with various concentrations of oestrogen. Maximal activation of the HeLa cells was achieved at an agonist concentration of 1 nM which resulted in approximately a 25-fold induction of luciferase activity compared with the control. At 10 nM of 17-β-oestradiol, the inducibility of the reporter gene system was greatly reduced (Fig. 1A). This phenomenon is precipitated by the excessive and prolonged stimulation of the oestrogen receptor by an agonist, which in turn results in the down regulation of the receptor (Xie *et al.*, 1999). However, significant induction of luciferase activity was observed at a concentration of agonist as low as 0.1 pM.

Analysis of the phytoestrogen content of Bupleurum & Peony Formula

A 4% stock solution of the Bupleurum & Peony Formula (lot number 7061180) was prepared and sterilized.

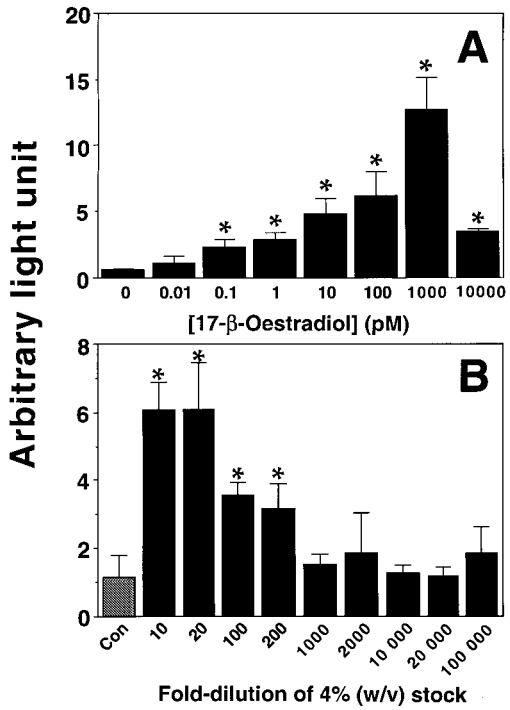

Figure 1. Induction of the luciferase activity of HeLa cells by 17-β-oestradiol and the Bupleurum & Peony Formula. HeLa cells were treated with (A) different concentrations of 17-β-oestradiol or (B) different dilutions of a 4% stock solution of the Bupleurum & Peony Formula (lot number 7061180) for 24 h. The cells were then assayed for the induction of luciferase activity. Control (Con), cells without drug treatment. *The luciferase activity was significantly higher than that obtained in the absence of drug treatment; $p < 0.05$, Bonferroni *t*-test.

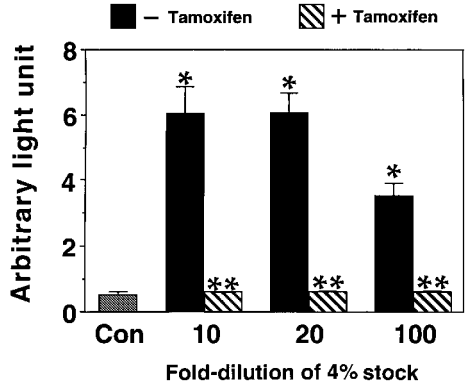

Figure 2. Tamoxifen blocks the induction of luciferase activity by the Bupleurum & Peony Formula. HeLa cells were treated with different dilutions 4% stock solution of the Bupleurum & Peony Formula (lot number 7061180) with or without 30 μM tamoxifen for 24 h. Control (Con), cells without drug treatment. * The luciferase activity was significantly higher than that obtained with the control; ** 30 μM tamoxifen significantly inhibited the luciferase activity in cells stimulated with the Bupleurum & Peony Formula; $p<0.05$, Bonferroni *t*-test.

Dilutions, ranging from 10- to 100 000-fold, of the stock solution were assayed for their ability to induce the chimeric receptor/reporter gene elements in a stably transfected HeLa cell line. As shown in Fig. 1B, maximal activation of the chimeric receptor/reporter gene system was achieved at a 20-fold dilution of the 4% stock. At this dilution, induction of bioluminescence by phytoestrogens was 4 orders of magnitude over that measured for the control. Even at a 200-fold dilution of the 4% stock, luciferase induction was approximately 2 orders of magnitude greater than that of the control. Thus, a detectable difference in luciferase induction, compared with the control, was obtained at concentrations of the formula as low as 0.02% (w/v).

Similar results were obtained with two separate lots of the Bupleurum & Peony Formula (lot number 7061180 and 710028024). At 20-fold dilution of 4% stock solutions, approximately a 4-fold increase in light emissions was observed in both lots compared with the controls (Table 1). Additionally, a third lot of the Bupleurum & Peony Formula (lot number 711148024) was also tested. Interestingly, this lot induced the reporter gene assay by a factor of approximately 8-fold compared with the control, and which was twice the value of induction observed with either of the previous lots of test formula. In contrast, no bioluminescence was detected with the crude glycoside extract of Qing Yang Shen (*Cynanchum otophyllum Schneid*), which contains otophyllosides A and B (Mu *et al.*, 1984) and lacks phytoestrogens (Table 1). In order to determine the specificity of the chimeric receptor/reporter gene elements to phytoestrogenic compounds, HeLa cell were pretreated with the oestrogen receptor antagonist, tamoxifen, prior to being challenged with the formula. Pretreatment of the cells with 30 μM tamoxifen completely abolished the formula's ability to activate the chimeric receptor (Fig. 2). This blockade of receptor activation by tamoxifen was observed for all dilutions of the 4% stock solutions tested.

Table 1. Differential induction of luciferase activity by the Bupleurum & Peony Formula and Qing Yang Shen

	Luciferase activity (ALU) (fold-induction)		
Drug treatment	Con	20-fold dilution	200-fold dilution
Bupleurum & Peony Formula			
Lot 7061180	1.17 ± 0.66	6.05 ± 1.62 (4.2)[a]	3.15 ± 0.74 (1.7)[a]
Lot 710028024	1.19 ± 0.43	5.62 ± 1.09 (3.7)[a]	3.67 ± 0.94 (2.1)[a]
Lot 711148024	1.73 ± 0.87	15.31 ± 1.87 (7.8)[a]	5.18 ± 0.22 (1.9)[a]
Qing Yang Shen	1.94 ± 0.87	1.96 ± 0.77 (0.0)	2.48 ± 1.25 (0.2)

HeLa cells were treated with either 20- or 200-fold dilutions of 4% stock solutions of the Bupleurum & Peony Formula (three different production lots) or Qing Yang Shen for 24 h. Control (Con), cells without drug treatment. Values shown in parenthesis indicate the fold-induction of luciferase activity. [a] The luciferase activity was significantly higher than that obtained with the control; $p < 0.05$, Bonferroni *t*-test.

DISCUSSION

In the present study we utilized an oestrogen-chimeric receptor/Gal4-response element regulated/luciferase-reporter gene (ER-Gal4-Luc) assay to assess its potential to serve as a robust bioassay system for the detection of phytoestrogens in TCMs. In assessing the assay, three criteria were used to determine its suitability for identifying oestrogen mimetic compounds present in complex TCM formulas. The three criteria included the assay's overall sensitivity, responsiveness and specificity to known phytoestrogens and the prototypic oestrogen, 17-β-oestradiol. Since the total phytoestrogen content of most TCM formulas is expected to be low, a robust bioassay is essential for their unambiguous detection. The results of the present study support the routine use of this system in assessing the phytoestrogen content of complex herbal formulas such as those commonly prescribed in TCM. Using the oestrogen-responsive chimeric receptor/reporter gene constructs stably transfected into HeLa cells, we showed that the bioassay possessed sub-nanomolar sensitivity to 17-β-oestradiol that resulted in approximately a 25-fold induction in luciferase-derived bioluminescence. Furthermore, expression of the chimeric receptor was shown to be sensitive to high concentrations of oestrogen, which led to its down-regulation and a significant reduction in bioluminescence. The overall sensitivity of this bioassay has been shown to depend upon receptor expression levels (Webb *et al.*, 1992). Therefore, receptor expression levels in the stably transfected HeLa cells should be periodically measured if this assay were to be used for future standardization of TCMs.

The TCMs used is this study were Bupleurum & Peony Formula and a total glycoside fraction of the plant *Cynanchum otophyllum Schneid* (CO total glycoside). Where the Bupleurum & Peony formula is a complex formulation consisting of ten different herbs (some of which are known to contain trace quantities of known phytoestrogens), the CO total glycoside is a TCM fraction that contains two primary chemical compounds, otophylloside A and B (two suspected steroidal-derived anticonvulsants with no known *in vitro* oestrogenic activity). Employing the current chimeric receptor-based bioassay, we showed that the system could readily detect the presence of phytoestrogens in dilute solutions of the complex Bupleurum & Peony Formula. Under the experimental condition used in these studies, a 20-fold dilution of the 4% stock solution formula resulted in approximately a 4-fold induction of bioluminescence for two of the lots tested, while a formula concentration of these different lots as low as 0.02% (w/v) was capable of stimulating a response twice the level of the control. Moreover, it is widely acknowledged that concentrations of various secondary metabolites in plants can vary greatly depending upon factors such as the geographical location of cultivation and the time of harvest. Although these details were not available for the Bupleurum & Peony Formula, it is note worthy that one of the lots tested appeared to possess approximately twice the phytoestrogen content of the other two samples. When applied to product quality control measures, the robustness of the reporter gene assay will ensure that an acceptable level of phytoestrogen activity is present in a particular product, and its sensitivity may also provide insights on the product's overall efficacy. Lastly, and as expected, CO total glycoside was not capable of eliciting an oestrogenic response and thus served as a negative control in the present study.

The final criterion to be assessed was the ER-Gal4-Luc bioassay's responsiveness to the oestrogen receptor antagonist, tamoxifen. Regardless of the source of oestrogen agonist used (17-β-oestrodiol or Bupleurum & Peony), pretreatment of the cells with tamoxifen resulted in a total inhibition of the assay to respond to oestrogenic stimulation. Thus, our studies show that the ER-Gal4-Luc bioassay system possesses exquisite oestrogenic sensitivity, responsiveness and specificity required for the accurate assessment of the phytoestrogen content of complex TCMs.

Several *in vivo* and *in vitro* assays have been developed to detect the oestrogenic properties of compound and complex mixtures. Among the various *in vivo* end points used for assessing oestrogen mimetics, the most common are cellular differentiation, enzymatic activity, marker protein expression, and changes in organ weights (Branham *et al.*, 1993; Heppell *et al.*, 1995; Medlock *et al.*, 1993; Teng, l995; Copper *et al.*, 1993; Lan and Katzenellenbogen, 1976; Lyttle and DeSombre, 1977). In contrast, *in vitro* assays associated with the detection of oestrogenic substances are based on well-characterized mechanisms of actions possessing more definable end points. Currently, there are approximately six such assay systems that have been developed to detect activities associated with alleged oestrogenic substances. The methodologies utilized in these systems are diverse which accounts for the various strategies employed in their development. The current group of bioassays associated with the detection of exogenous oestrogen-like activities include: (i) competitive oestrogen receptor binding (Miksicek, 1993); (ii) cell proliferation (Soto *et al.*, 1995); (iii) foci formation assay; (iv) protein expression assay (Jordan *et al.*, 1985): (v) oestrogen response element-regulated reporter genes (Miksicek, 1995); (vi) chimeric receptor/reporter genes (Connor *et al.* (1995): and (vii) yeast-based assays (Pierrat, *et al.*, 1992). Previously, Zacharewski published a critical review (Zacharewski, 1997) that detailed the advantages and disadvantages of the various *in vitro* bioassays systems currently available for identifying oestrogenic substances.

Generally, *in vitro* oestrogenic assay systems are preferred over *in vivo* systems for numerous reasons, such as reduced cost and ease of manipulation. The competitive ER binding, cell proliferation, foci formation and protein expression assays all possess various drawbacks that hamper their sensitivities, responsiveness, specificities and/or routine utility. Although the competitive ER binding assay is extensively employed to study receptor–ligand interactions they are not capable of predicting if such interactions are sufficient to elicit an oestrogenic response (Katzenellenbogen and Gorski, 1975). Likewise, utilization of cell proliferation as a surrogate end point, although attractive for its simplicity, is known to be affected by a number of factors that influences its overall sensitivity and specificity. Some of these factors include: (i) culture conditions (Karey and Sirbasku, 1988; Berthois, *et al.*, 1986): (ii) receptor and/or cell densities (Horowitz *et al.*, 1978; Page *et al.*, 1983): (iii) serum composition (Johnson *et al.*, 1989; Page *et al.*, 1983); (iv) clonal heterogeneity (Graham *et al.*, 1990). As

a result of its sensitivity (estrogen EC_{50} ~50 pM) and responsiveness (>20-fold), the foci formation assay is an attractive bioassay system for detecting the presence of phytoestrogens in complex mixtures such as TCMs. However, the greatest drawback of this assay is the substantial incubation period (9 to 10 days) required for foci development. With respect to protein expression assays, it usually requires burdensome and/or protracted methodologies for their assessment. Furthermore, other non-oestrogenic mechanisms may be elicited that either induce or repress the target end point, which in turn would greatly increase the potential number of false positives and/or negatives associated with using this approach. Although, we have mentioned oestrogenic yeast-based bioassays for the sake of presenting the full repertoire of assays currently available for assessing oestrogenic activities, we will not consider the assay further due to several unique differences in its ligand specificities compared with mammalian models (Zysk *et al.*, 1995; Kohno *et al.*, 1994).

Recombinant receptor/reporter-based assays can overcome many of the shortcomings of non-recombinant-based assays. As a result of their outstanding responsiveness, sensitivity and specificity, they are extremely useful in comparative studies designed to address the relative potency of agonist and antagonist targeting the recombinant receptor of interest. The selection of an appropriate reporter gene is based primarily on its stability and ease of detection, and thus, is subsequently engineered into the assay to provide a reliable and sensitive surrogate end point. Other added benefits of such assays over more conventional biochemical analyses lie in the fact that utilization of a living system provides a means to assess potential synergistic, antagonistic, or other dynamic interactions that may exist between compounds or arise in complex mixtures. In selecting which recombinant receptor/reporter gene assay to use in the present study, we eventually employed the ER- Gal4-Luc assay (Ponglikitmongkol *et al.*, 1990) for assessing the phytoestrogen content of the two TCMs. This assay system possesses sub-nanomolar sensitivity in either transiently transfected MCF-7 or stably transfected HeLa cells with an associated response factor of 40- to 50-fold and 8- to 12-fold, respectively. Several studies have shown that the difference in responsiveness between the two cell lines is most probably due to differences in recombinant receptor expression levels (Webb, *et al.*, 1992). Since the chimeric receptor fuses the ligand binding domain of the oestrogen receptor to the DNA binding domain of a yeast transcriptional factor, Gal4, and there are no known mammalian homologues of Gal4, induction of the reporter gene (luciferase) is mediated exclusively by the activation of the chimeric receptor. One other important consideration for selecting this system is that the binding affinity and ligand specificities of the chimeric oestrogen receptor do not differ from those of the endogenous receptor (Evans, 1988; Kumar, *et al.*, 1987).

Unlike the other bioassays that have been developed for assessing exogenous oestrogenic compounds, the chimeric receptor/reporter gene assay possesses the unique advantage of allowing the detection of variations in oestrogen responsiveness, sensitivity and specificity that exists between the oestrogen receptors encoded by different species. This aspect of the assay is relevant for the purposes of assessing the presence of phytoestrogen in TCMs that may possess potential veterinarian applications. Additionally, the ER-Gal4-Luc bioassay system has previously been used to assess the oestrogen receptor-mediated activities of complex mixtures such as urban air particulate matter, and samples of effluent and black liquor generated from a paper processing company (Zacharewski *et al*., 1995; Balaguer *et al.*, 1996; Clemons *et al.*, 1996). Exposure to such exogenous oestrogenic compounds has been shown to adversely effect laboratory animals in terms of their endocrine and reproductive systems. The abnormalities in the reproductive fitness and the development of cancers resulting from exposure to these compounds are believed to invoke similar mechanisms in humans and fauna.

In order for TCMs to gain international acceptance, both their safety and efficacies will require scientific validation. Utilizing a cell-based assay, described herein, certain medicines prescribed for menopausal syndrome and premenstrual symptoms maybe initially assessed for phytoestrogens, and in the process may provide insights into potential mechanisms of actions for these TCMs.

In conclusion, the results of this study suggest that the Bupleurum & Peony Formula has measurable phytoestrogen content, and a HeLa cell line-based, ER-Gal4-Luc assay can be developed to a quick, sensitive, and quantifiable bioassay for detecting the presence of phytoestrogen in complex TCM formulas.

Acknowledgements

The financial or material donations from the Hong Kong Industry Department (Industrial Support Fund AF/178/97) and Sun-Ten Pharmaceutical Co. Limited, Taiwan, are gratefully acknowledged.

REFERENCES

Balaguer P, Joyeux A, Denison M, Vincent R, Gillesby B, Zacharewski T. 1996. Assessing the estrogenic and dioxin-like activities of chemicals and complex mixtures using *in vitro* recombinant receptor-reporter gene assays *Can J Physiol Pharmacol* **74**: 216–222.

Berthois Y, Katzenellenbogen J A, Katzenellenbogen BS. 1986. Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. *Proc Natl Acad Sci USA* **8**: 2496–2500.

Branham WS, Zehr DR, Sheehan DM. 1993. Estrogen receptors in human nontarget tissues: biological and clinical implications. *Proc Soc Exp Biol Med* **203**: 297–303.

Ciocca DR, Roig LMV. l995. Failure of chloro-S-triazine-derived compounds to induce estrogen receptor-mediated responses *in vivo* and *in vitro*. *Endocrine Rev* **16**: 35–62.

Clemons JH, Marvin CR, McCarry BE, Allan L, Bryant DW, Zacharewski TR. 1996. *Abstracts of the l7th Annual Meeting of the Society of Environmental Toxicology and Chemistry*, Washington, DC, November 17-21, 1996; Abstracts of 490; Society of Environmental Toxicology and Chemistry: Pensacola, FL, 211.

Connor K Howell J, Chen L *et al*. 1995. Failure of chloro-S-triazine-derived compounds to induce estrogen receptor-mediated responses *in vivo* and *in vitro*. *Fund Appl Toxicol*, **30**: 93–101.

Copper RL Goldman JM Vandenbergh JG. 1993 *Monitoring of the Estrous Cycle in Laboratory Rodent by Vaginal Lavage*. Academic Press: San Diego, **3**: 45–56.

Evans R. 1988. The steroid and thyroid hormone receptor superfamily. *Science* **240**: 889–895.

Graham ML II, Keett NL, Miller LA *et al*. 1990 T47DCO cells, genetically unstable and containing estrogen receptor mutations, are a model for the progression of breast cancers to hormone resistance. *Cancer Res* **50**: 6208–6217.

Heppell SA, Denslow ND, Folmar LC, Sullivan CV. 1995. Universal assay of vitellogenin as a biomarker for environmental estrogens. *Environ Health Perspect* **103(Suppl. 7)**: 9–15.

Horowitz KB, Zava DT, Thilager AK, Jensen E, McGuire WL. 1978. Steroid receptor analyses of nine human breast cancer cell lines. *Cancer Res* **38**: 2434–2437.

Johnson MD, Westley BR, May FEB. 1989. Oestrogenic activity of tamoxifen and its metabolites on gene regulation and cell proliferation in MCF-7 breast cancer cells. *Br J Cancer* **59**: 727–738.

Jordan VC, Koch R, Bain RR. 1985. *Prolactin Synthesis by Cultured Rat Pituitary Cells: An Assay to Study Estrogens, Antiestrogens and Their Metabolites In Vitro*. Elsevier: New York.

Karey KP, Sirbasku DA. 1988. Differential responsiveness of human breast cancer cell lines MCF-7 and T47D to growth factors and 17 beta-estradiol. *Cancer Res* **48**: 4083–4092.

Katzenellenbogen BS, Gorski J. 1975. *Estrogen Actions on Syntheses of Macromolecules in Target Cells*. Academic Press: New York, **3**: 187–243.

Kohno H, Gandini O, Curtis SW, Korach KS. 1994. Anti-estrogen activity in the yeast transcription system: estrogen receptor mediated agonist response. *Steroids* **59**: 572–578.

Kumar V, Green S, Stack G, Berry M, Jin JR, Chambon P. 1987. Functional domains of the human estrogen receptor. *Cell* **51**: 941–951.

Lan NC, Katzenellenbogen BS. l976. Temporal relationships between hormone receptor binding and biological responses in the uterus: studies with short- and long-acting derivatives of estradiol. *Endocrinology* **98**: 220–227.

Lyttle CR, DeSombre ER. 1977. Uterine peroxidase as a marker for estrogen action. *Proc Natl Acad Sci USA* **74**: 3162–3166.

Mayr U, Butsch A, Schneider S. 1992. Validation of two *in vitro* test systems for estrogenic activities with zearalenone, phytoestrogens and cereal extracts. *Toxicology* **74**: 135–149.

Medlock KL, Forrester TM, Sheehan DM. 1993. Progesterone and estradiol interaction in the regulation of rat uterine weight and estrogen receptor concentration. *Proc Soc Exp Biol Med* **205**: 146–153.

Miksicek RJ. 1993. Commonly occurring plant flavonoids have estrogenic activity. *Mol Pharmacol* **44**: 37–43.

Miksicek RJ. 1995. Estrogenic flavonoids: structural requirements for biological activity. *Proc Soc Exp Biol Med* **208**: 44–50.

Mu QZ, Lu J, Zhou QL. 1984. Two new antiepilepsy compounds: otophyllosides A and B. *Scientia Sinica* **3**: 34–40.

Page MJ, Field JK, Everett NP, Green CD. 1983. Serum regulation of the estrogen responsiveness of the human breast cancer cell line MCF-7. *Cancer Res* **4**: 1244–1250.

Pierrat B, Henry D, Lemoine Y, Losson R. 1992. Functional analysis of the human estrogen receptor using a phenotypic transactivation assay in yeast. *Gene* **119**: 237–245.

Ponglikitmongkol M, White J, Chambon P. 1990. Synergistic activation of transcription by the human estrogen receptor bound to tandem responsive elements. *EMBO J* **9**: 222l–2231.

Rosenblum ER, Stauber RE, Van Thiel DH, Campbell IM, Gavaler JS. 1993. Assessment of the estrogenic activity of phytoestrogens isolated from bourbon and beer. *Alcoholism: Clin Exp Res* **17**: 1207–1209.

Soto AM, Lin T-M, Sakabe K, Olea N, Damassa DA, Sonnenschein C. 1995. Variants of the human prostate LNCaP cell line as tools to study discrete components of the androgen-mediated proliferate response. *Oncology Research* **7**: 545–548.

Strom SS, Yamammura Y, Duphorne CM *et al*. 1999. Phytoestrogen intake and prostate cancer: a case–control study using a new database. *Nutr Cancer* **33**: 20–25.

Teng C. 1995. Mouse lactoferrin gene: a marker for estrogen and epidermal growth factor. *Environ Health Perspect* **103(Suppl. 7)**: 17–20.

Webb P, Lopez GN, Greene GL, Baxter JD, Kushner PJ. l992. The limits of the cellular capacity to mediate an estrogen response. *Mol Endocrinol* **6**: 157–167.

Xie T, Ho SL, Ramsden D. 1999. Characterization and implications of estrogenic down-regulation of human catechol-O-methyltransferase gene transcription. *Mol Pharmacol* **56**: 31–38.

Zacharewski T. 1997. *In vitro* bioassays for assessing estrogenic substances. *Environ Sci Technol* **31**: 613–622.

Zacharewski T, Berhane K, Gillesby BE. 1995. Detection of estrogen- and dioxin- like activity in pulp and paper mill black liquor and effluent using *in vitro* recombinant receptor/reporter gene assays. *Environ Sci Technol* **29**: 2140–2146.

Zysk JR, Johnson B, Ozenberger BA, Bingham B, Gorski J. 1995. Selective uptake of estrogenic compounds by *Saccharomyces cerevisiae*: a mechanism for antiestrogen resistance in yeast expressing the mammalian estrogen receptor. *Endocrinology* **136**: 1323–1326.

