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Construct and validation of progesterone-responsive luciferase reporter plasmid to detect progestogenic and anti-progestogenic activity

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Abstract

Chemical and immunological analyses used to detect progesterone and its agonists and antagonists are unable to detect biological activities of unknown compounds. The objective of the experiment was to develop a progesterone-responsive reporter plasmid to detect progestogens and anti-progestogens. Progesterone-responsive luciferase reporter plasmid, pGL3-2PRE-TATA, was constructed by inserting two progesterone-response elements (PRE) and the TATA box at the multiple cloning site of pGL3 basic vector. T47D human breast cancer cells were co-transfected with c pGL3-2PRE-TATA and pCH110 plasmid. The transfected cells were exposed to progesterone, anti-progesterone, herb extracts or animal serum to induce the luciferase gene expression. Cells were lysed, and cell lysates were used to measure the luciferase and β-galactosidase expression level as well as total protein content. Luciferase activity was normalized by β-galactosidase activity and total protein content. The constructed plasmid was sensitive to progestogens and anti-progestogens in a dose-responsive manner. The pGL3-2PRE-TATA plasmid can be used to detect the progestogenic and anti-progestogenic activity of known and unknown compounds. (*Bangl. vet*. 2013. Vol. 30, No. 1, 10 – 19)

Introduction

Progesterone is a steroid hormone involved in the establishment and maintenance of pregnancy (Graham and Clarke, 1997; Lessey, 2003) the reproductive cycle and mammary gland development (Lydon *et al.,* 1995; Aupperlee *et al.,* 2005). Progesterone also has functional roles in repair of myelin, regeneration of axon and neuroprotection (Schumacher *et al.,* 2012). Sources of progesterone are ovaries (after ovulation), adrenal gland and placenta. Progesterone levels are lower in men and postmenopausal women than cyclic women (NIH, 2011) and gradually increase during pregnancy. Synthetic progestogens exert similar effects to progesterone, although their chemical structure, efficiency and pharmacokinetic properties differ (Stanczyk, 2003; Sitruk-Ware, 2004). Animal, plant (Iino *et al*., 2007) and synthetics are the three sources of progestogen. Some steroid hormones, chemicals and environmental wastes have progestogenic or anti-progestogenic activity (Chatterjee *et al.,* 2008). Progestogens are widely used in hormone replacement therapy, contraception, treatment of cancer and of reproductive disorders, such as endometriosis and uterine adenomyosis (Spitz and Chwalisz, 2000; Kuhl and

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Wiegratz, 2004; Jaakkola *et al.*, 2011). Anti-progesterone (Mifepristone, RU486) blocks progesterone, and can be used for termination of early pregnancy (Hazra and Pore, 2001).

Progesterone exerts its effect on target tissue through genomic or non-genomic pathway (Baldi *et al.,* 2009). In genomic pathway, progesterone exerts its effect through intracellular receptors (Lydon *et al.,* 1995; Baldi *et al.,* 2009), which binds to progesterone-response elements in regulatory regions of specific genes (Beato, 1989; Smith & O'Malley 2004). In non-genomic pathway, progesterone alters the level of second messenger (Baldi *et al.,* 2009) via membrane-bound progesterone receptor (Luconi *et al.,* 1998 and Jang and Yi, 2005). Intracellular progesterone receptors express as isoforms PR-A and PR-B. Both are transcribed from a single gene by two distinct promoters and two translation initiation signals (AUG) (Mulac-Jericevic *et. al,* 2003). PR-B contains an additional NH_2-terminal stretch of about 165 amino acids. The trans-activating ability of PR-A is less strong than PR-B, and PR-A is a transcriptional suppressor (Schoonen *et al*., 1998).

Steroid-like synthetic chemicals are used as animal growth promoters (Ismail *et al.,* 1995; Badr *et al.,* 2010), plant growth enhancers (Uthairatanakij, 2007) pesticides and herbicides (Andersen *et al*., 2002). Heavy metallic and biochemical substances are used in plastic, leather and pharmaceutical industry (Osinubi and Saalu, 2009). Some chemicals affect the reproductive system of wildlife and humans (Vos *et al.,* 2000; Sharpe, 2001) by inhibiting the steroid hormone from binding to its receptor (Hotchkiss *et al*., 2009).

Several assays have been developed to measure progesterone and anti-progesterone activities. The McPhail assay (McPhail, 1934) is a good *in vivo* assay to evaluate progesterone-induced effects on rabbit endometrium, but needs live animals. Enzyme-linked immunosorbent assay (ELISA) and radio-immuno assay (RIA) are commonly used, but are not able to detect biological activities of unknown compounds. Therefore, it is necessary to develop a new assay to detect the total progestogenic or anti-progestogenic activity of compounds. The objective of the study was to develop a progesterone-responsive luciferase reporter plasmid to detect progesterone and its agonists and antagonists.

Material and Methods

DNA vectors, chemicals and reagents

The pGL3 basic and pSV-β-galactosidase control vector, luciferase enzyme assay system, β-galactosidase enzyme assay system and restriction enzymes were purchased from Promega (Madison, WI, USA). Progesterone, oestradiol-17β, testosterone, dexamethasone, melengesterone acetate (MGA), altrenogest and mifepristone (RU486) were bought from Sigma Aldrich (St. Louis, MO, USA). Lipofectamine 2000, Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA).

Construction of reporter plasmid pGL3-2PRE-TATA

The reporter plasmid pGL3-2PRE-TATA was constructed in two steps. First, two 104-mer oligomers, antisense to each other, were synthesized by incorporating a minimal promoter, two progesterone-response elements upstream of the promoter, and *Hind*III restriction site at both ends. These oligomers were annealed by gradually reducing the temperature from 99°C to 25°C to synthesize a double-stranded DNA. In the second step, 86-bp DNA fragment was inserted into the *Hind*III restriction site of pGL3 basic vector to construct the recombinant plasmid, pGL3-2PRE-TATA (Fig. 1).

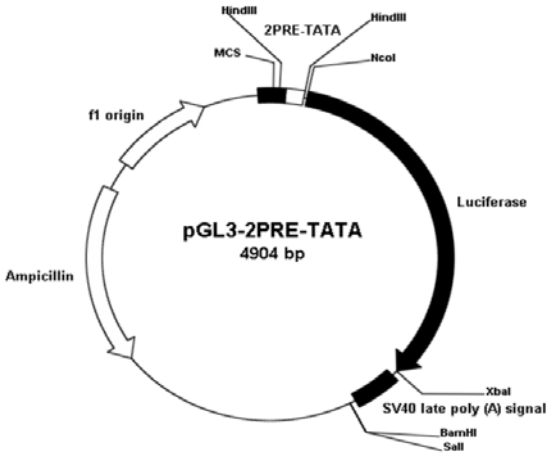

Fig. 1. Diagram of the constructed pGL3-2PRE-TATA plasmid. Two progesterone-response elements (PRE) and the TATA box were inserted at the multiple cloning site of pGL3 basic vector

Cell culture

Human breast adenocarcinoma cell line, T-47D, was purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in DMEM supplemented with 1% sodium pyruvate, 3.7 g/L sodium bicarbonate, and 10% FBS. For assay, cells were maintained in a phenol red-free DMEM supplemented with 1% sodium pyruvate and 10% dextran-coated charcoal absorbed FBS or 10% castrated pig serum. All cells were cultured at 37°C and 5% CO_2 in a humidified incubator.

Cell transfection and progestogenic activity assay

Two days before transfection, T47D cells were plated at 2.5×10^5 cells per well into six well plates with DMEM containing 2% dextran-coated charcoal FBS and incubated for 48 hours. Cells were co-transfected with 2 μg of pGL3-2PRE-TATA and 1 μg of pSV-β-galactosidase control vector using lipofectamine 2000, according to manufacturer's protocol. The transfected cells were treated with different concentrations of progesterone, anti-progesterone, other steroids, LC extract or 10% pig's serum. After 24 hours, cells were lysed and the cell lysates were used to determine luciferase and β-galactosidase activities by β-galactosidase and luciferase assay kits according to the manufacturer's instructions. Protein contents were determined by Bradford protein assay kit (Bio-Rad, Hercules, CA, USA). Luciferase activity was normalized by β-galactosidase activity and protein content.

Preparation of Ligusticum chuanxiong extracts

Rhizome *Ligusticum chuanxiong* (LC) roots, purchased from Sun Ten Pharmaceutical Co. Ltd. (Taichung, Taiwan), were ground and soaked with 100% ethanol for 72 hours at room temperature. The extracts were filtered and kept inside a hood for rapid evaporation of ethanol. Dried extracts were diluted at the stock concentration and kept at -80°C.

Animal experiment design

Four barrows Landrace-Duroc × Yorkshire with an average age of 6 to 8 months and average bodyweight of 120 kg were housed separately. Control was fed normal commercial feed, positive control supplemented with 20 mg altrenogest and treatment pig supplemented with 100g ethanolic extract of LC. Blood was collected from jugular vein before and after feeding. Samples were allowed to clot on ice and centrifuged at 1500 ×g for 30 minutes at 4°C and kept at -20°C.

Data analysis

Luciferase activity in each well was measured as relative light units (RLU). All data were summarized from at least three independent experiments with triplicates. Fold induction was calculated by dividing the mean luciferase activity from different treatments (progesterone, anti-progesterone, other hormones, hormone-like substances, herbal extracts, and pig serum) with control wells. Data were analyzed by using Statgraphics software (STATGRAPHICS Centurion XVI, Warrenton, VA, USA). Each value is presented as the mean ± SEM. Mean values were compared by analysis of variance (ANOVA) with LSD tests for comparing group. A significant level of 0.05 was adopted.

Results and Discussion

Response to progesterone and its agonist and antagonist

Plasmid pGL3-2PRE-TATA was highly sensitive to progesterone in a dose-dependent manner (Fig. 2). The minimal and maximal luciferase activities were found when cells were exposed to 1 nM and 1 μM of progesterone, respectively (Fig. 2). The maximal induced luciferase activity was 19.7 times that in vehicle control (Fig. 2). The minimal detectable luciferase activity was 1.98 times that in vehicle control (Fig. 2). There was no difference between more than 1 μM and 1 μM progesterone-induced luciferase activities. The induced luciferase activities of 1 μM MGA and altrenogest were 18 and 17 times that in vehicle control, respectively (Fig. 3) but not significantly different from that induced by 1 μM progesterone. To validate sensitivity to progesterone receptor antagonist, transfected cells were exposed to 100 nM RU486 in presence of 100 nM progesterone. Fig. 4 demonstrates that RU486 blocked the progesterone.

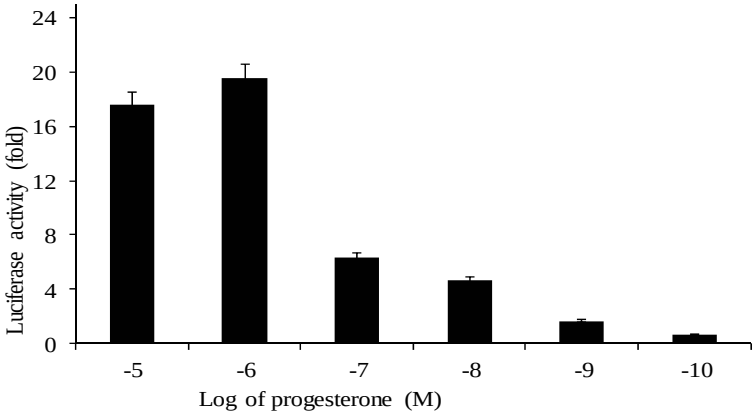

Fig. 2. Dose-dependent response of constructed plasmid to progesterone. Data represented as the mean fold induction compared to vehicle control ± SEM

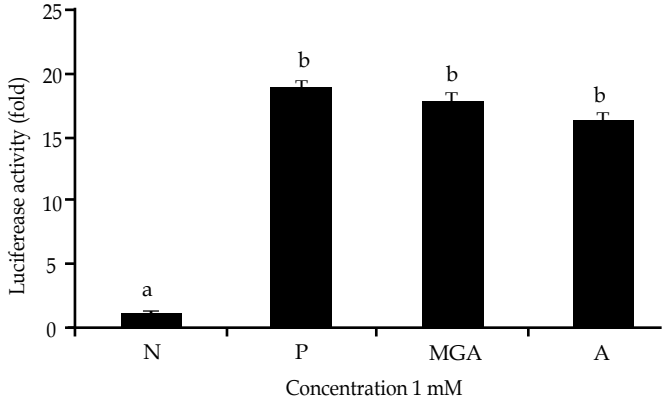

Fig. 3. Response to progesterone agonist of the constructed plasmid, negative control (N), progesterone (P) melengesterone acetate (MGA) and altrenogest (A). Data represented as the mean fold induction compared to vehicle control ± SEM. Different letters denote significant difference ($P<0.05$)

Ligand specificity

To investigate the ligand specificity of the recombinant plasmid pGL3-2PRE-TATA, transfected cells were exposed to oestrogen, testosterone, and dexamethasone. Concurrently cells were exposed to progesterone and ethanol as positive and vehicle control, respectively. Fig. 5 shows that 100 nM oestrogen, testosterone, and dexamethasone failed to induce the luciferase activity compared to vehicle control.

Response to LC extract

LC extract from 20 to 40 µg induced a dose-response increase in luciferase activity. LC extract 50 µg or above induced a dose-responsive decrease in luciferase activity. At maximal concentration of 40 µg LC extract, luciferase activity was 7 times that in vehicle control and was equivalent to 100 nM progesterone-induced activity (Fig.s 2 and 6).

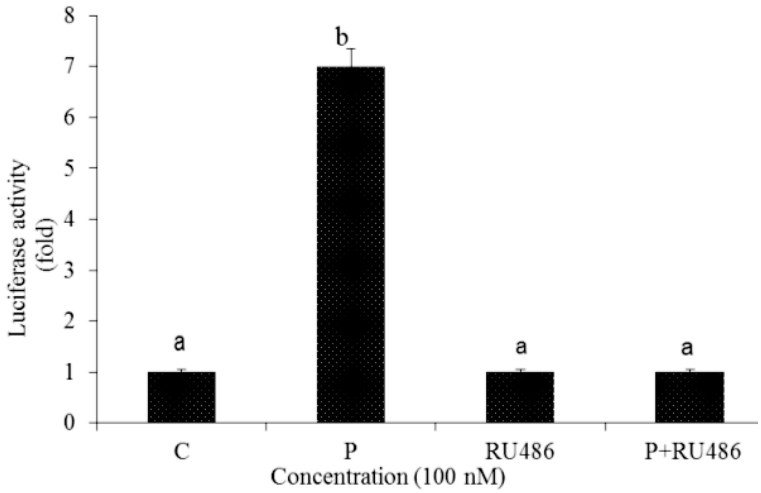

Fig. 4. Response to anti-progesterone (RU486), Control (C) and Progesterone (P). Data represented as the mean fold induction compared to vehicle control ± SEM. Different letters denote significant difference (P<0.05)

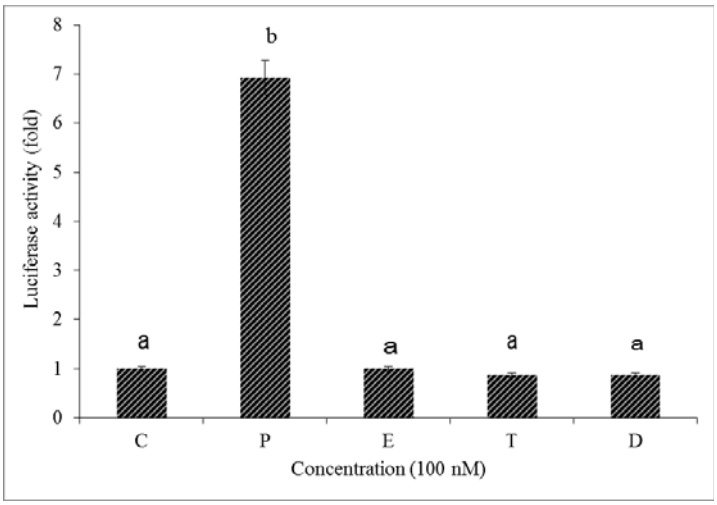

Fig. 5. Ligand specificity of the constructed plasmid, Control (C), Progesterone (P), Oestrogen (E), Testosterone (T), and Dexamethasone (D). Data represented as the mean fold induction compared to vehicle control ± SEM. Different letters denote significant difference (P<0.05)

Response to animal serum

Castration of pigs largely eliminate endogenous source of progesterone. To validate the response to serum progesterone, castrated pigs were fed progestogen (altrenogest) and phytoprogesterone containing herb extract (LC) and serum was separated after collecting blood. Transfected cells were incubated for 24 hours with 10% pig sera. Fig. 7 shows that progestogenic activities of serum from LC extract-fed and altrenogest-fed animal were higher than control. Therefore, the constructed plasmid was sensitive to animal serum progestogen.

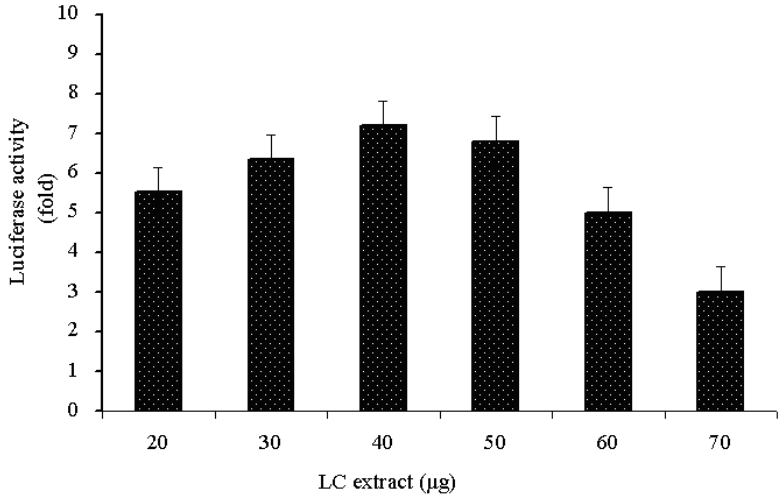

Fig. 6. Dose-dependent progestogenic activity of LC extract. Data represented as the mean fold induction compared to vehicle control ± SEM.

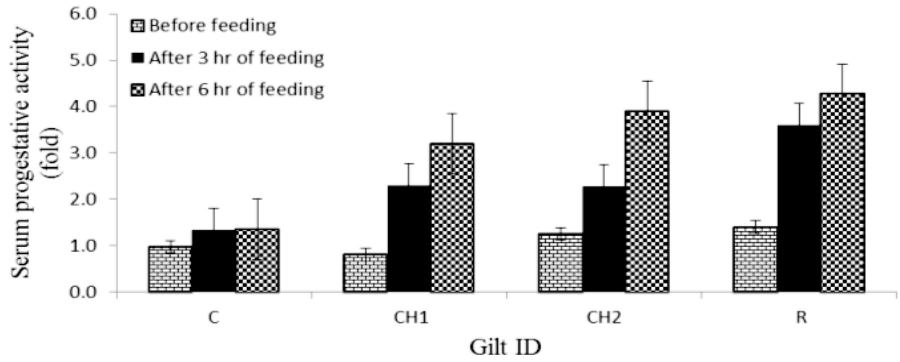

Fig. 7. Serum progestogenic activities of castrated pigs. Serum of control (C), LC extract-fed (CH1 and CH2) and altrenogest-fed (R) pigs. Data were presented as the mean fold induction compared to vehicle control ± SEM

In this study, a highly sensitive progesterone-responsive plasmid was developed to detect progesterone and its agonists and antagonists. Animal, plant and synthetic sources of progestogen were used to test the responsiveness of the plasmid. Constructed plasmid was highly responsive to progesterone (Fig. 2), pig serum (Fig. 7) and plant extract (Fig. 6). The minimal detectable level was 1 nM progesterone. The plasmid pGL3-2PRE-TATA was sensitive enough to be used to detect the physiological status. Oestrogen, testosterone and dexamethasone did not significantly induce luciferase activity (Fig. 5). Although, all steroid hormone receptors belong to the same superfamily (Lydon *et al.*, 1995) RU486 suppressed the progestogenic activity by blocking the binding of progesterone to its receptor (Hazra and Pore, 2001). Fig. 4 showed that RU486 suppressed 100 µM progesterone-induced luciferase activities. The developed plasmid was also responsive to anti-progesterone. T47D, a human breast cancer cell line was chosen because it naturally expresses both

progesterone receptors A and B. Progesterone and its agonists and antagonists bind to nuclear progesterone receptor (Lydon *et al.*, 1995) to form receptor-ligand complex, which binds to progesterone response elements on pGL3-2PRE-TATA plasmid to induce luciferase expression.

As a practical validation of plasmid, ethanolic extracts of LC and progestogen and sera from castrated pigs fed LC extract were used. LC contains dimeric phyto-progestogens, such as 3, 8-dihydro-diligustilide and Riligustilide (Lim *et al.*, 2006a). Ethanolic extract of LC induced progestogenic activity in a dose-dependant and biphasic manner (Fig. 6). The plasmid can, therefore, be used to screen plants for progestogenic and anti-progestogenic activity. Serum progestogenic activities were significantly higher in castrated pigs after feeding altrenogest and LC extract (Fig. 7). Similar result was reported by Lim *et al.* (2006b) who found the serum progestogenic activity was significantly elevated after oral administration of LC extract in rats. This result indicates that exogenous progestogen can be monitored with the plasmid.

Conclusions

It is concluded that the plasmid pGL3-2PRE-TATA can be used for screening progestogenic and anti-progestogenic compounds from single or mixed samples.

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