

Danggui Buxue Tang – A Chinese herbal decoction activates the phosphorylations of extracellular signal-regulated kinase and estrogen receptor α in cultured MCF-7 cells

Qiu T. Gao^a, Roy C.Y. Choi^a, Anna W.H. Cheung^a, Judy T.T. Zhu^a, Jun Li^a, Glanice K.Y. Chu^a, Ran Duan^a, Jerry K.H. Cheung^a, Zhi Y. Jiang^a, Xiao B. Dong^a, Kui J. Zhao^{a,b}, Tina T.X. Dong^a, Karl W.K. Tsim^{a,*}

^a Department of Biology and Center for Chinese Medicine R&D, The Hong Kong University of Science and Technology, Clear Water Bay Road, Hong Kong, China

^b Beijing Friendship Hospital, Affiliate of Capital University of Medical Sciences, 95 Yong An Road, Beijing 100050, China

Received 29 September 2006; revised 7 December 2006; accepted 7 December 2006

Available online 14 December 2006

Edited by Lukas Huber

Abstract Danggui Buxue Tang (DBT), a Chinese herbal decoction used to treat ailments in women, contains Radix Astragali (Huangqi; RA) and Radix Angelicae Sinensis (Danggui; RAS). The weight ratio of RA to RAS used in DBT must be 5:1 as stipulated as early as AD 1247; however, DBT's mechanism of action has never been described. Here, the estrogenic effects of DBT were investigated by determining the phosphorylations of estrogen receptor α (ER α) and extracellular signal-regulated kinase 1/2 (Erk1/2) in cultured MCF-7 cells. The application of DBT triggered the phosphorylation of ER α and Erk1/2 in a time-dependent manner. In contrast to the effect of estrogen, DBT triggered ER α phosphorylation at both S118 and S167. This DBT-specific phosphorylation was not triggered by an extract of one of the individual herbs, or by mixing the extracts of RA and RAS. DBT-induced downstream signals are described here. These signals suggest the uniqueness of this Chinese herbal decoction that requires a well-defined formulation. © 2006 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Chinese medicine; Erk; Estrogen receptor; Phosphorylation; Estrogenic effects; Signaling

1. Introduction

Women in menopause suffer from hot flashes, sweating, anxiety, mood swings, as well as an increased risk for many health problems, such as bone density reduction, cardiovascular disease and osteoporosis. These problems are largely due to the deficiency of ovarian hormones, especially estrogen [1]. Hormone replacement therapy (HRT) was introduced to allay menopausal symptoms 20 years ago [2]. However, HRT recently became a subject of debate because subject-based studies revealed an increased risk of breast cancer and coronary

artery disease in women who use HRT [3,4]. In view of these clinical risks, extensive efforts have been devoted to developing different preparations that would yield the benefits of hormone therapy but with minimal inconvenience or risk [5]. Herbal medicines are promising preparations and lead to fewer side effects [6]. Indeed, herbal products have become increasingly popular in the last decade and are being used widely by women in relieving their menopausal symptoms [7].

Among thousands of herbal formulae from traditional Chinese medicine (TCM), Danggui Buxue Tang (DBT; a herbal decoction) is a simple combination of two herbs. DBT was first described in *Neiwaishang Bianhuo Lun* by Li Dongyuan in China in AD 1247. Li described the DBT formula that included: 10 *qian* of Radix Astragali (RA), roots of *Astragalus membranaceus* (Fisch.) Bunge or *Astragalus membranaceus* (Fisch.) Bunge var. *mongholicus* (Bunge) P.K. Hsiao, and two *qian* of Radix Angelicae Sinensis (RAS), roots of *Angelica sinensis* (Oliv.) Diels. A *Qian* was the weight unit in ancient China; one *qian* equals about 3 g. The mixed herbs were boiled in two bowls of water over a moderate heat until the final volume was reduced by half. Traditionally, DBT has been prescribed to women in China as a remedy for menopausal symptoms. These women are directed to drink DBT daily to raise their “*Qi*” (their vital energy) and nourish their “Blood” (their body circulation).

Pharmacological results indicated that DBT has the abilities to promote hematopoietic functions, to stimulate cardiovascular circulation, to prevent osteoporosis, to increase anti-oxidation activity and to stimulate the immune system [8,9]. By determining the chemical and biological properties of DBT, the optimized extraction conditions have been established [8,10], which, interestingly, are in accord with the weight ratio of 5:1 for RA to RAS in the ancient preparation.

The rationale for including different herbs in Chinese herbal decoctions has never been fully explained, which consequently hinders the development of multi-herb decoctions as disease and disorder remedies. In a pioneering effort to reveal the downstream signaling mechanisms of DBT, we employed human breast cancer MCF-7 cell as a model system to determine the estrogenic activity of DBT via the activation of gene transcription. Indeed, the estrogenic effects are the primary function of the DBT decoction. In addition, we revealed the

*Corresponding author. Fax: +852 2358 1559.
E-mail address: botsim@ust.hk (K.W.K. Tsim).

Abbreviations: DBT, Danggui Buxue Tang; RA, Radix astragali; RAS, Radix angelicae sinensis; ER, estrogen receptor; Erk, extracellular signal-regulated kinase

underlying signaling pathways of DBT by the phosphorylations of estrogen receptor α (ER α) and extracellular signal-regulated kinase 1/2 (Erk1/2).

2. Materials and methods

2.1. Plant materials and preparation of DBT

Fresh roots were obtained from China in September to October of 2002. We collected three-year-old *A. membranaceus* var. *mongholicus* roots from Shanxi province and two-year-old *A. sinensis* roots from Minxian in Gansu province. These areas had been demonstrated to produce the best quality RA [11] and RAS [12], respectively. Preparation of the herb materials under optimized conditions was described previously [10]. The corresponding vouchers as forms of whole plants, voucher # 02-9-1 for *A. membranaceus* var. *mongholicus* and voucher # 02-10-4 for *A. sinensis*, were deposited in the Chinese medicinal plant collection in the Department of Biology, The Hong Kong University of Science and Technology, China. In preparing the DBT, exact amounts of RAS and RA were weighed according to a ratio of 5:1 and then mixed well in a vortex. The mixture was boiled in 8 volumes of water (*v/w*) for 2 h and extracted twice; this extraction followed the ancient recipe that had been shown to have the best extracting conditions [10]. Separate samples of RAS and RA were extracted by the same method. The extracts were dried by lyophilization and stored at -80°C .

2.2. HPLC fingerprinting of DBT

Ferulic acid was purchased from Sigma (St. Louis, MO), calycosin, formononetin and ligustilide (*z*-isoform) were kindly provided by Prof. Pengfei Tu, the Medical College of Peking University; their purities, confirmed by HPLC, were higher than 99.0%. AR- and HPLC-grade reagents were from Merck (Darmstadt, Germany). A Waters (Milford, MA) HPLC system consisting of a 600 pump, a 717 auto-sampler and a UV/VIS Photodiode Array 2996 Detector was used for all analyses. Chromatographic separations were carried out on a DELTA-PAK C₁₈ column (particle size 4.6 μm , 3.9 mm \times 150 mm) with acetonitrile (as Solvent A) and 0.01% phosphoric acid (as Solvent B) in the mobile phase at a flow rate of 1.0 ml/min at room temperature. A linear gradient elution was applied from 15% to 65% of Solvent A starting from 0 to 60 min. Samples were filtered through a 0.45 μm Millipore syringe filter unit. Twenty microliter samples were injected for HPLC analysis.

2.3. The MCF-7 cell culture

Human mammary epithelial carcinoma cell line MCF-7 was obtained from American Type Culture Collection (ATCC) and was grown in modified Eagle's medium (MEM), supplemented with 10% fetal bovine serum (FBS), 1 mM nonessential amino acids, 0.1 mM sodium pyruvate, 100 U/ml penicillin, and 100 $\mu\text{g/ml}$ streptomycin in a humidified CO₂ (5%) incubator at 37 $^{\circ}\text{C}$. The culture reagents were purchased from Invitrogen Technologies (Carlsbad, CA). Before plating, PBS was used to wash the cells, and the medium was changed to MEM- α without phenol red containing 5% charcoal dextran-treated FBS for 1 day. MCF-7 cells were seeded in a 24-well plate at 25000 cells/cm². Three hours before the drug treatment, the medium was changed to phenol-red free MEM- α . The cells were treated with DBT and other samples for appropriate times at a final concentration of 1 mg/ml. In the blocking analyses, ER antagonist ICI 182780 (Tocris, Ellisville, MO) at 0.1 μM and Erk1/2 inhibitors PD098059 (20 μM) and U0126 (10 μM ; Sigma) were applied 3 h before the application of other drugs. The positive control was 17 β -estradiol (0.1 μM ; Sigma), dissolved in dimethyl sulfoxide (DMSO), or comparisons. Phorbol 12-myristate 13-acetate (TPA; Sigma) at 0.1 μM was used as the Erk1/2 activator.

2.4. Estrogen promoter assay in the MCF-7 cells

Three repeats of estrogen responsive elements (ERE: 5'-GGT CAC AGT GAC C-3') were synthesized as described previously [13] and then subcloned into a promoter-reporter vector called pTAL-Luc (Clontech, Mountain View, CA) that has a downstream reporter of the firefly luciferase gene; this DNA construct was named pERE-

Luc. Cultured MCF-7 cells were transfected with pERE-Luc to generate stable cells according to a previous report [14]. To determine the estrogenic properties, DBT or another extract was applied to the cultures for 2 days. If necessary, the cultures were pretreated with ICI 182780, PD098059 and U0126 for 3 h before the drug application. Afterward, the medium was aspirated, and MCF-7 cells were washed by cold PBS. The cells were lysed with 100 μl /well of lysis buffer containing 0.2% Triton X-100, 1 mM dithiothreitol and 100 mM potassium phosphate buffer (pH 7.8) at 4 $^{\circ}\text{C}$. Following centrifugation at 14000 rpm and 4 $^{\circ}\text{C}$ for 10 min, the supernatant was collected, and 50 μl of the supernatant was used to perform the luciferase assay (Tropix Inc., Bedford, MA); the activity was normalized by equal amounts of protein. The cell viability of the cultured MCF-7 cells was measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay according to the manufacturer's instructions by measuring the absorbance at 570 nm.

2.5. Determination of ER α and Erk 1/2 phosphorylation in MCF-7 cells

The phosphorylations of ER α at serine 118 (S118) and serine 167 (S167), and Erk1/2 were determined by western blot assays. Cultures were serum starved for 3 h before the drug applications. After drug treatments, including all the inhibitors or activators, the cultures were collected immediately in lysis buffer (125 mM Tris-HCl, 2% SDS, 10% glycerol, 200 mM 2-mercaptoethanol, pH 6.8) and the proteins were subjected to SDS-PAGE analysis. After transferring the proteins to membranes, the membranes were incubated with anti-phospho-ER α S118 (Upstate, Lake Placid, NY) at 1:2000 dilution, anti-phospho-ER α S167 at 1:1000 dilution (Upstate), or anti-phospho-Erk1/2 (Cell Signaling, MA, USA) at 1:5000 dilution at 4 $^{\circ}\text{C}$ for 12 h. Following incubation in horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies in 1:5000 dilutions for 1 h at room temperature, the immuno-complexes were visualized by the enhanced chemiluminescence (ECL) method (Amersham Biosciences, Piscataway, NJ). The band intensities in the control and agonist-stimulated samples, run on the same gel and under strictly standardized ECL conditions, were compared on an image analyzer, using in each case a calibration plot constructed from a parallel gel with serial dilutions of one of the samples.

2.6. Other assays

The protein concentrations were measured routinely by Bradford's method with a kit from Bio-Rad Laboratories (Hercules, CA). Statistical tests were run on the PRIMER program, version 1 (Primer of Biostatistics); differences from the basal or control values (as shown in the figures) were classed as significant [*] where $P < 0.05$, more significant [**] where $P < 0.01$ and highly significant [***] where $P < 0.001$.

3. Results

3.1. Standardization of DBT

DBT, composed of RA and RAS in a weight ratio of 5:1, was prepared according to the optimized extraction conditions as described previously [8,10]. In order to standardize the herbal extract chemically, we generated HPLC fingerprints. Fig. 1 shows a typical HPLC fingerprint of DBT at an absorbance of 254 nm. By discovering the amounts of two chemical markers in RA (calycosin and formononetin) and two others in RAS (ferulic acid and ligustilide), we were able to standardize the weight ratio of 5:1 for optimal DBT. We found that standardized DBT should contain 0.186 mg calycosin, 0.155 mg formononetin, 0.351 mg ferulic acid and 0.204 mg ligustilide per one gram dried weight of DBT. From the extraction efficiency calculations, we found that about 1.92 g of DBT was obtained from 5 g RA mixed with 1 g RAS; the yield of DBT in this weight ratio was $32 \pm 3\%$ ($n = 5$). These parameters established the chemical standards and the optimal DBT mixture for the remaining experiments.

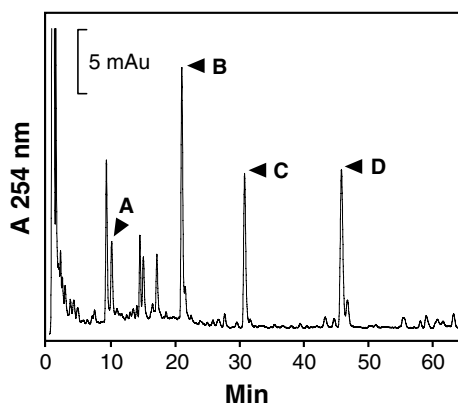


Fig. 1. Chemical standardization of DBT by HPLC fingerprint analysis. In the HPLC fingerprint at an absorbance of 254 nm, the peaks corresponding to ferulic acid (A), calycosin (B), formononetin (C) and ligustilide (D) were identified. The details of the marker identification were described previously [11]. These chemical characterizations are used to identify the standardized DBT for all the biochemical analyses. A typical fingerprint of DBT is shown, $n = 5$.

3.2. Estrogenic properties of DBT

A promoter-reporter construct (pERE-Luc; see Fig. 2b upper panel) containing three repeats of estrogen-responsive element was stably transfected into MCF-7 cells, and this stable cell line was used to determine the estrogenic effect of DBT. Different amounts of DBT were applied to the cultures for 2 days. Two biological effects were subsequently determined: the cell viability and the promoter-driven luciferase activity.

As shown in Fig. 2a upper panel, RA, RAS and DBT were not able to alter the mitogenic activity of MCF-7 cells in terms of cell proliferation, even at high concentrations of DBT (data not shown). In contrast, application of 17 β -estradiol showed a strong cell proliferation effect on cultured MCF-7 cells, which indicated a distinct cell proliferation effect of DBT as compared to estrogen. In the estrogen-regulated promoter activity assay, different herbal extracts were added to stably pERE-Luc-transfected MCF-7 cells. As expected, 17 β -estradiol at 0.1 μ M, as a control, caused a 3-fold increase in the promoter activity. The application of 1 mg/ml DBT led to activation of the promoter, and the potency was the strongest among the different herbal extracts we tested (Fig. 2a lower panel). Application of 1 mg/ml RA or 1 mg/ml RA + RAS (boiled separately and then mixed together) also induced promoter activity; however, the activity was lower when compared to the activity of DBT (Fig. 2a lower panel). One milligram per milliliter RAS did not have an activation effect. The DBT-induced pERE-Luc activity was similar after either 1 or 2 days of treatment. In addition, the DBT treatment showed a dose-dependent response in activating the pERE-driven luciferase activity (Fig. 2b lower panel). The pretreatment of ICI 182780, an estrogen antagonist, fully blocked the response.

3.3. Phosphorylation of ER α by DBT

ER α belongs to a super family of ligand-activated transcription factors, whose transcriptional activities are influenced by various signaling messengers [15]. ER α can be phosphorylated on multiple amino acid residues including S104, S106, S118, S167, etc. Among all these possible phosphorylation sites,

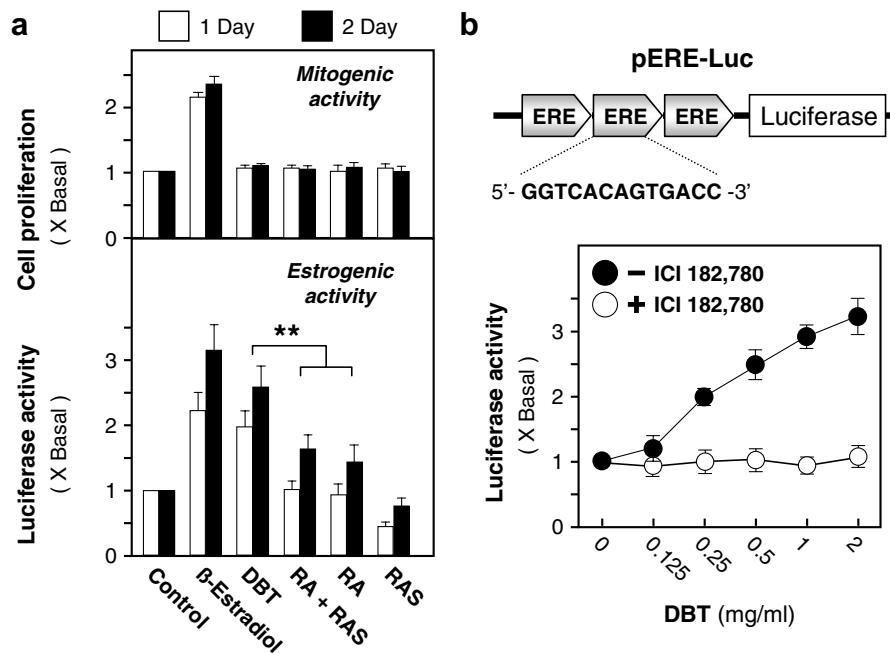


Fig. 2. DBT stimulates the transcriptional activity of the estrogen-responsive element but not the cell proliferation in MCF-7 cells. (a) Mitogenic and estrogenic activities by DBT. pERE-Luc stably transfected MCF-7 cultures were treated with DBT, RA, RAS and RA + RAS extracts (1 mg/ml) for 1 or 2 days and then were used to perform cell proliferation assays (MTT assay; upper panel) and luciferase assays (lower panel). β -Estradiol (0.1 μ M) was used as a positive control. Values of cell proliferation and promoter-driven luciferase (pERE-Luc) activity are expressed as the ratio to the basal reading where the control (without drug) equals to 1. ** $P < 0.01$ as compared with different groups. (b) pERE-Luc was constructed by three repeats of ERE in a luciferase promoter-reporter vector (upper panel). The pERE-Luc stably transfected MCF-7 cells were treated with DBT at different doses for 1 day (lower panel), and then the luciferase assay was performed (lower panel). ICI 182780 (0.1 μ M) pretreatment blocked the effect of DBT. Data are expressed as means \pm S.E.M., where $n = 4$, each with triplicate samples.

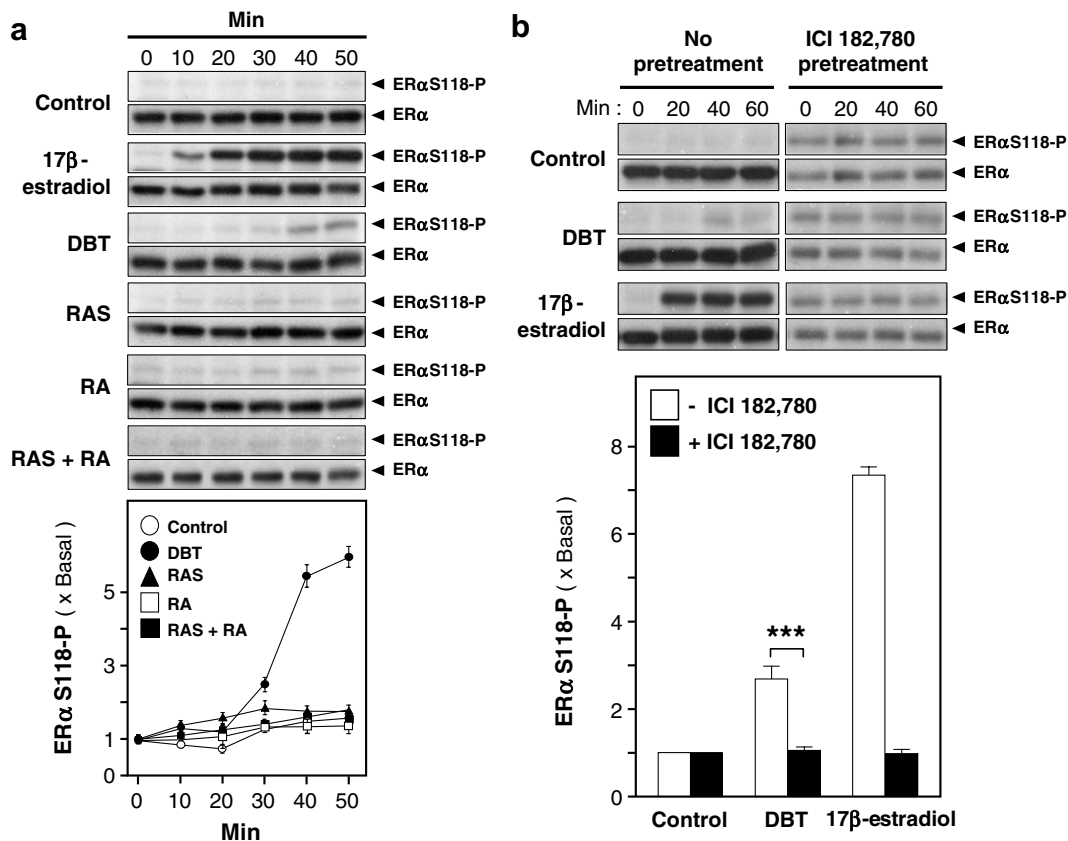


Fig. 3. DBT induces the phosphorylation of ER α on S118. (a) MCF-7 cultures were serum starved for 3 h before the addition of DBT, RA, RAS and RA + RAS extracts (1 mg/ml) for different durations. β -Estradiol (0.1 μ M) was used as a positive control. Total ER α and S118 phosphorylated ER α (ER α S118-P), both at \sim 66 kDa, were revealed by using specific antibodies. The lower panel shows the quantitation of phosphorylation from the blots by calibrating the densitometer. (b) Cultures were pretreated with ICI 182780 at 0.1 μ M for 3 h before the drug application. The inductive effect of DBT on ER α phosphorylation (S118) was blocked by ICI 182780. The lower panel shows the quantitation of phosphorylation from the blots at 60 min by calibrating the densitometer. Phosphorylation values are expressed as the ratio to the basal reading where time 0 (untreated as basal) equals to 1, and in means \pm S.E.M., where $n = 4$. *** $P < 0.001$ as compared to the control group.

S118 is the major phosphorylation site for the binding of estrogen. Application of 17 β -estradiol onto cultured MCF-7 cells induced the phosphorylation of ER α at S118 (\sim 66 kDa); the phosphorylation started 10 min after the drug application and it lasted for at least 50 min (Fig. 3a). The phosphorylation of S118 in ER α was also triggered by DBT, and this phosphorylation lasted for at least 50 min (over fivefold activation) after the drug treatment (Fig. 3a). In contrast, extracts derived from RA alone, RAS alone, or RA + RAS induced S118 phosphorylation to a small extent with a 2-fold increase in the activity.

ICI 182780 belongs to a class of 'pure' anti-estrogen drugs [16], which act as potent antagonists by inducing rapid receptor turnover rates and blocking the agonistic activity both in vivo and in vitro [17]. The pure antagonistic property of ICI 182780 comes from its steroidal structure containing a long bulky side chain that induces a distinct conformational change in the ligand binding domain of ER [18]. Cultured MCF-7 cells were pretreated with the antagonist (0.1 μ M) for 3 h before drug treatments. Results indicated that the pretreatment of ICI 182780 completely blocked ER α phosphorylation at S118 that had been induced by DBT and 17 β -estradiol (Fig. 3b). The application of ICI 182780 induced the basal S118 phosphorylation of ER α , and the application was concomitant with down regulation of total ER α levels

(Fig. 3b). These results agree with the results of a previous report [19].

In addition to S118 phosphorylation, we also investigated phosphorylation at S167 in ER α . Although the requirement of S167 for estrogen activation remains controversial, the phosphorylation of this serine residue provides evidence to support the activation of ER α , as well as its function, in regulating gene transcriptions [20]. In cultured MCF-7 cells, application of 17 β -estradiol or the extracts derived from RAS, RA, RAS + RA did not induce ER α phosphorylation at S167. Surprisingly, DBT induced a specific activation at S167; the activation increased by over 15-fold after the drug challenge (Fig. 4a). The DBT-specific ER α phosphorylation of S167 was fully blocked through the pretreatment with ICI 182780 (Fig. 4b), suggesting a possible interaction of ER α with DBT.

3.4. Phosphorylation of Erk1/2 by DBT

Mitogen-activated protein kinases (MAPKs) are involved in numerous cellular responses including cell growth and differentiation, and they have been shown to participate in ER α phosphorylation. We chose to study the phosphorylation of Erk1/2, an MAPK with a role in the classical Raf-MEK-Erk pathway. Similar to our analysis of ER α phosphorylation, we treated serum-starved MCF-7 cultures with different drugs and col-

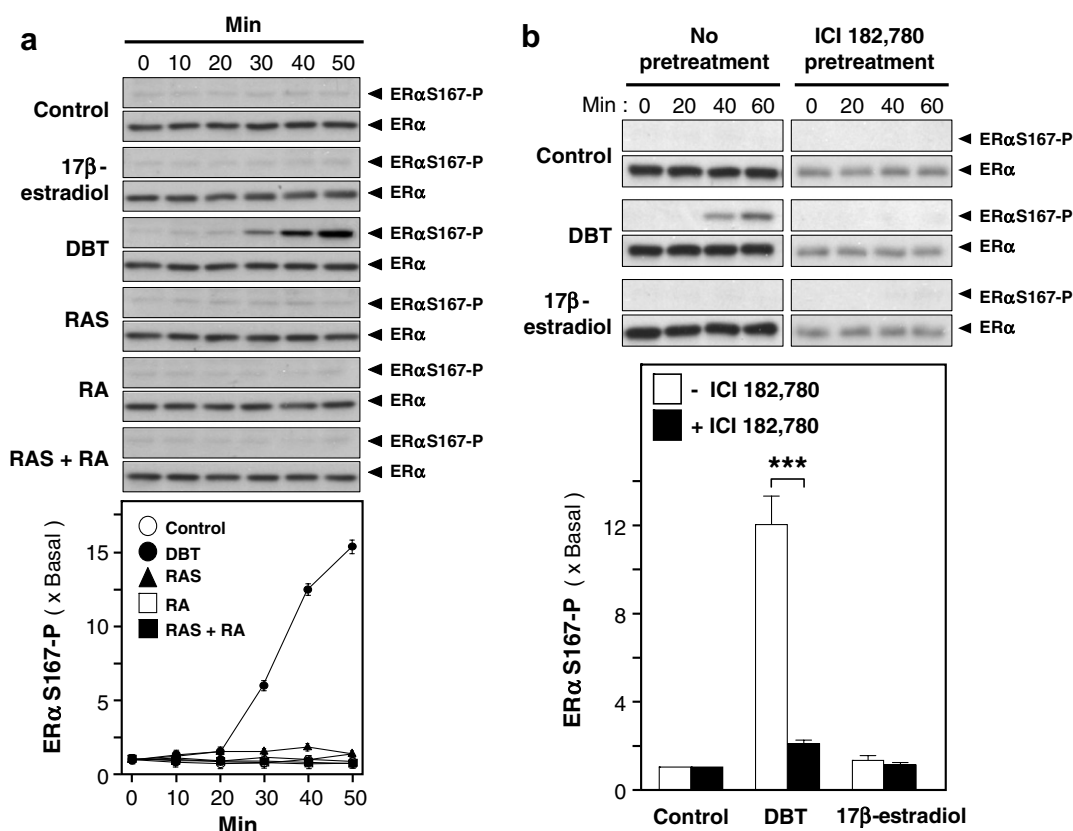


Fig. 4. DBT induces the phosphorylation of ER α on S167. (a) MCF-7 cultures were serum starved for 3 h before the addition of DBT, RA, RAS and RA + RAS extracts (1 mg/ml) for different durations. Total ER α and S167 phosphorylated ER α (ER α S167-P) were revealed by using specific antibodies. β -Estradiol (0.1 μ M) was used as a positive control. The lower panel shows the quantitation of the phosphorylation from the blots by calibrating the densitometer. (b) The inductive effect of DBT on ER α phosphorylation (S167) was blocked by ICI 182780 (0.1 μ M) pretreatment. The lower panel shows the quantitation of phosphorylation from the blots at 60 min by calibrating the densitometer. Phosphorylation values are expressed as the ratio to the basal reading where time 0 (untreated as basal) equals to 1, and in means \pm S.E.M., where $n = 4$. *** $P < 0.001$ as compared to the control group.

lected the cells at different times. The phosphorylations of Erk1 (~44 kDa) and Erk2 (~42 kDa) were markedly increased with addition of DBT with the maximal effect of about 15-fold at 30 min after treatment. The activation was maintained longer than 50 min after the start of the treatment (Fig. 5a). The application of 17 β -estradiol, which served as a control, induced Erk1/2 phosphorylation at an extent similar to that of DBT. In addition, the phosphorylation of Erk1/2 was insignificant when MCF-7 cells were treated with RA, RAS, or the mixture of RA + RAS (Fig. 5a).

We further confirmed the role of Erk1/2 in activating ER α . In cultured MCF-7 cells, the application of TPA (12-*O*-tetradecanoylphorbol 13-acetate), a known activator of Erk1/2, induced the phosphorylation of Erk1/2 and ER α at both S118 and S167 residues (Fig. 5b). The time courses of these activations were very similar, i.e., the phosphorylations started after 15 min of TPA treatment and reached a maximum at about 30 min (Fig. 5b). These results showed that Erk1/2 could serve as an upstream activator of ER α .

Two specific MAPK inhibitors, PD098059 and U0126, were used to test the specificity of the DBT effects. In the cultured cells, both of the inhibitors fully blocked the DBT-induced Erk1/2 phosphorylation (Fig. 6a and b). However, the inhibitors only partially blocked the DBT-induced ER α phosphorylation at the S118 and S167 residues (Fig. 6a and b). The effects

of PD098059 and U0126 in blocking the DBT-induced ER α phosphorylation did not change even when the amount of the inhibitors was increased (data not shown), which suggested that the effect of DBT in phosphorylating ER α was not fully explained by Erk1/2 only. The amounts of total Erk and ER α did not change. We suggest two possible pathways to account for the DBT-induced ER α phosphorylation: (1) Erk-dependent and (2) Erk-independent signaling pathways. In line with this suggestion, we observed that the MAPK inhibitors only partially blocked the DBT-induced estrogenic activity in pERE-Luc- transfected MCF-7 cells (Fig. 6c).

4. Discussion

By analyzing the downstream signaling pathway(s) of DBT-induced estrogenic effects, we are able to provide evidence of the uniqueness of a specific combination of RA and RAS in creating the Chinese medicinal DBT. In addition, the estrogenic activation of DBT is very distinct from that of estrogen as shown in the specific phosphorylation of ER α at S167 in DBT-treated breast cancer cells. Although the activity of the estrogen-responsive element in pERE-Luc stably expressing MCF-7 cells could be activated by extracts of individual herbs, or by a mixture of RA and RAS, the phosphorylations of ER α

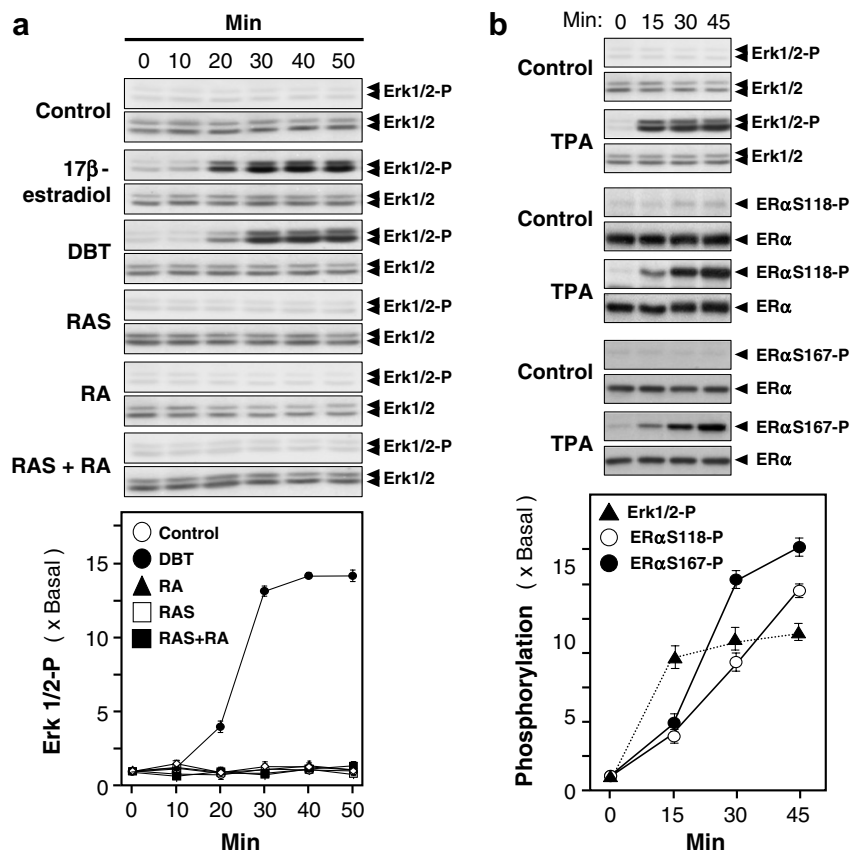


Fig. 5. DBT and TPA induce Erk1/2 and ER α phosphorylation. (a) MCF-7 cultures were serum starved for 3 h before the addition of DBT, RA, RAS and RA + RAS extracts (1 mg/ml) for different times. Total and phosphorylated Erk1/2 were revealed by using specific antibodies. β -Estradiol (0.1 μ M) was used as a positive control. The lower panel shows the quantitation of phosphorylation from the blots by calibrating the densitometer. (b) Cultured MCF-7 cells were treated with 0.1 μ M TPA for 45 min and then collected to determine the protein phosphorylation with antibodies against S118 and S167 phosphorylated ER α and phosphorylated Erk1/2. Total proteins were served as the loading control in all cases. The lower panel shows the quantitation of phosphorylation from the blots by calibrating the densitometer. Phosphorylation values are expressed as the ratio to the basal reading where time 0 (untreated as basal) equals to 1, and in means \pm S.E.M., where $n = 4$.

at S167 and of Erk1/2 were found only in DBT-treated cultures. The specific DBT-induced phosphorylations, therefore, suggested that boiling the two herbs together is essential; this method of preparation of DBT, indeed, has long been recommended by TCM practitioners in China. In addition to the study with MCF-7 cells, the role of the correct weight ratio of RA to RAS has also been demonstrated in cultured osteoblasts [8] and T-lymphocytes [9]. Interestingly, the biological properties of DBT as demonstrated here are in agreement with the preparation and usage of DBT as first described about 800 years ago.

Besides S167 phosphorylation, DBT also had distinct effects on S118 phosphorylation of ER α . Although both DBT and estrogen could phosphorylate ER α on S118, the phosphorylation profiles were different in time and magnitude. For instance, the phosphorylation induced by DBT started at 40 min after treatment with 5-fold greater activation, which was much slower and less intense than treatment with 17 β -estradiol. The distinctive effects of DBT on S118 and S167 phosphorylations might correlate with the inability of DBT to stimulate the proliferation of cultured MCF-7 cells. ER α could be phosphorylated by direct binding with estrogen, and/or the phosphorylation could be mediated by other signaling molecules. For instance, the phosphorylation of ER α S118

could be mediated by CDK7 in responding to 17 β -estradiol, which was also phosphorylated by Erk1/2 [21,22]. S167 of ER α is a target site for protein kinase B (Akt) and ribosomal S6 kinase (p90RSK) [23,24]. Additionally, both of the two kinases could be activated by Erk1/2 [24,25]. Therefore, the specific effect of DBT on Erk1/2 phosphorylation could possibly account for the ER α phosphorylations on S118 and S167, which subsequently stimulate the transcription of the estrogen-responsive genes. Thus, we suggest that the activation of ER α by DBT is different from a classical activation pathway mediated by estrogen, which requires further elucidation.

In agreement with the ancient formulation, the 800-year-old decoction described here with RA and RAS in a 5:1 ratio possesses the best biological activities in stimulating estrogenic responses [8] as well as immunological responses [9]. In agreement with our previous results, we have demonstrated the specific requirement of boiling the two herbs together during DBT preparation to achieve full biological activity. Why boiling the two herbs together leads to better outcomes remains an unanswered question. We offer two hypotheses to explain the unique biological functions of DBT. First, DBT might contain additional chemicals than those in the extracts of RA or RAS. Very likely, these additional chemicals are only soluble in DBT, i.e., the boiling of RA and RAS together en-

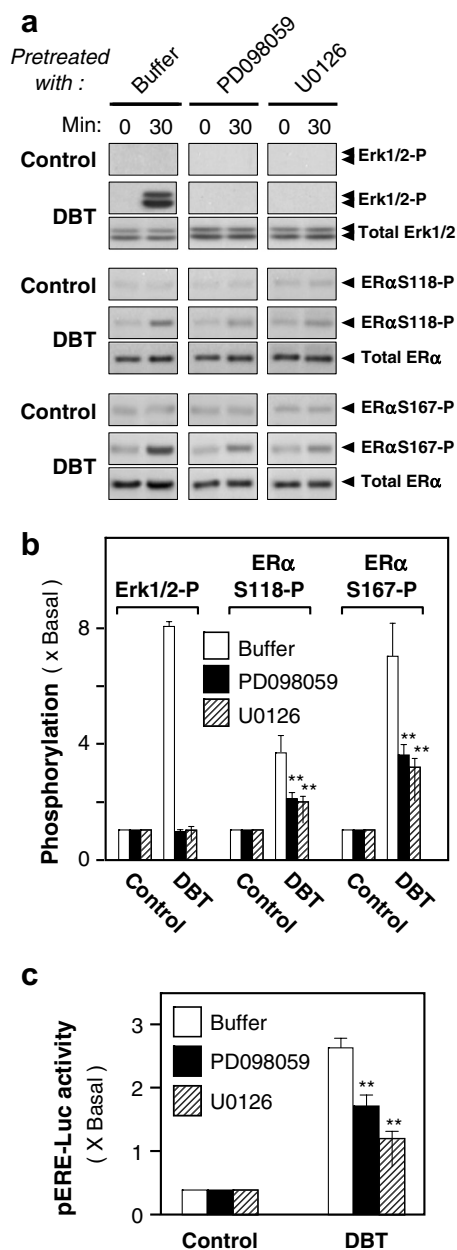


Fig. 6. The DBT-induced estrogenic effects are partially blocked by Erk1/2 inhibitors. (a) MCF-7 cultures were pretreated with buffer (0.1% DMSO), 20 μ M PD098059 and 10 μ M U0126 for 3 h before the addition of DBT (1 mg/ml) for 30 min. Cultures were collected to determine the ER α and Erk1/2 phosphorylations. Total proteins were served as the loading control in all cases. (b) The quantitation of phosphorylations from the blots in (a) was done by calibrating the densitometer. (c) The pERE-Luc transfected MCF-7 cultures were pretreated with inhibitors as in (a) before the addition of DBT for 2 days. Cultures were then collected to determine the luciferase activity. Phosphorylation values or the promoter-driven luciferase activities are expressed as the ratio to the basal or control reading where time 0 or untreated culture equals to 1, and in means \pm S.E.M., where $n = 4$. ** $P < 0.01$ as compared to the buffer control.

hances the solubilities of the chemicals. The additional chemicals could be responsible for the distinct DBT-specific effects. The optimized ratio of the two herbs in yielding more active ingredients can be a good explanation for the DBT-specific

effects. Our chemical analyses showed that higher amounts of RA-derived astragaloside IV, calycosin, formononetin, and RAS-derived ferulic acid were found in the DBT decoction; there was as much as double the amount of these chemicals in the 5:1 ratio than in other ratios of RA to RAS [8]. Second, there could be a synergistic effect of different components in DBT; this synergistic effect is not present in the extracts of the single herbs. Unfortunately, we do not have direct evidence to test these hypotheses from our DBT experiments. However, because of the failure of the RA + RAS mixture to perform the same functions as DBT, we believe that the second hypothesis is less likely.

It is well known that steroid hormones, especially estrogen, can induce cell proliferation, which subsequently may lead to an increase risk of developing breast cancer. Indeed, this issue is a major concern in the use of estrogen replacement therapy. In contrast, DBT did not alter the proliferation of MCF-7 cells, even at high concentrations. In accordance with this observation, patients with breast tumors with high phosphorylation on ER α S167 responded positively to endocrine therapy, and these patients had a better survival rate [26]. In view of the aforementioned characteristics, DBT could serve as a novel therapeutic treatment to replace estrogen to relieve menopausal symptoms.

Acknowledgements: This research was supported by grants from the University Grants Committee (AoE/B-10/01) and the Research Grants Council (HKUST6419/06 M) of the Hong Kong SAR and from Nanjing University of Traditional Chinese Medicine (FJK 2006011) to K.W.K.T. K.W.K.T. held a visiting professorship at the School of Pharmaceutical Sciences, Peking University in 2005.

References

- [1] Harlow, B.L. and Signorello, L.B. (2000) Factors associated with early menopause. *Maturitas* 35, 3–9.
- [2] Nichols, K.C., Schenkel, L. and Benson, H. (1984) 17 beta-estradiol for postmenopausal estrogen replacement therapy. *Obstet. Gynecol. Surv.* 39, 230–245.
- [3] Beral, V., Banks, E., Reeves, G. and Appleby, P. (1999) Use of HRT and the subsequent risk of cancer. *J. Epidemiol. Biostat.* 4, 191–210.
- [4] Timins, J.K. (2004) Current issues in hormone replacement therapy. *N. J. Med.* 101, 21–27.
- [5] Brzezinski, A. and Debi, A. (1999) Phytoestrogens: the ‘natural’ selective estrogen receptor modulators. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 85, 47–51.
- [6] Glazier, M.G. and Bowman, M.A. (2001) A review of the evidence for the use of phytoestrogen for traditional estrogen replacement therapy. *Arch. Intern. Med.* 16, 1161–1171.
- [7] Geller, S.E. and Kronenberg, F. (2003) Toward optimal health: the experts discuss the use of botanicals by women. *J. Womens Health (Larchmt)* 12, 847–852.
- [8] Dong, T.T.X., Zhao, K.J., Gao, Q.T., Ji, Z.N., Zhu, T.T., Li, J., Duan, R., Cheung, A.W. and Tsim, K.W.K. (2006) Chemical and biological assessment of a Chinese herbal decoction containing Radix Astragali and Radix Angelicae Sinensis: determination of drug ratio in having optimized properties. *J. Agric. Food Chem.* 54, 2767–2774.
- [9] Gao, Q.T., Cheung, J.K.H., Li, J., Chu, G.K.Y., Duan, R., Cheung, A.W.H., Zhao, K.J., Dong, T.T.X. and Tsim, K.W.K. (2006) A Chinese herbal decoction, Danggui Buxue Tang, prepared from Radix Astragali and Radix Angelicae Sinensis stimulates the immune responses. *Planta Med.* 73, 1227–1231.
- [10] Song, Z.H., Ji, Z.N., Lo, C.K., Dong, T.T., Zhao, K.J., Li, O.T., Haines, C.J., Kung, S.D. and Tsim, K.W. (2004) Chemical and biological assessment of a traditional Chinese herbal decoction prepared from Radix Astragali and Radix Angelicae Sinensis:

- orthogonal array design to optimize the extraction of chemical constituents. *Planta Med.* 70, 1222–1227.
- [11] Ma, X.Q., Shi, Q., Duan, J.A., Dong, T.T. and Tsim, K.W. (2002) Chemical analysis of *Radix Astragali* (Huangqi) in China: a comparison with its adulterants and seasonal variations. *J. Agric. Food Chem.* 50, 4861–4866.
- [12] Zhao, K.J., Dong, T.T.X., Tu, P.F., Song, Z.H., Lo, C.K. and Tsim, K.W.K. (2003) Molecular genetic and chemical assessment of *Radix Angelica* (Danggui) in China. *J. Agric. Food Chem.* 51, 2576–2583.
- [13] Klinge, C.M. (2001) Estrogen receptor interaction with estrogen response elements. *Nucleic Acids Res.* 29, 2905–2919.
- [14] Tung, E.K.K., Choi, R.C.Y., Siow, N.L., Jiang, J.X.S., Ling, K.K.Y., Simon, J., Barnard, E.A. and Tsim, K.W.K. (2004) P2Y2 receptor activation regulates the expression of acetylcholinesterase and acetylcholine receptor genes at vertebrate neuromuscular junctions. *Mol. Pharmacol.* 66, 794–806.
- [15] Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H., Metzger, D. and Chambon, P. (1995) Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science* 270, 1491–1494.
- [16] Parker, M.G. (1993) Action of “pure” antiestrogens in inhibiting estrogen receptor action. *Breast Cancer Res. Treat.* 26, 131–137.
- [17] McDonnell, D.P. (2005) The molecular pharmacology of estrogen receptor modulators: implications for the treatment of breast cancer. *Clin. Cancer Res.* 11, 871–877.
- [18] Pike, A.C., Brzozowski, A.M., Walton, J., Hubbard, R.E., Thorsell, A.G., Li, Y.L., Gustafsson, J.A. and Carlquist, M. (2001) Structural insights into the mode of action of a pure antiestrogen. *Structure* 9, 145–153.
- [19] Joel, P.B., Traish, A.M. and Lannigan, D.A. (1998) Estradiol-induced phosphorylation of serine 118 in the estrogen receptor is independent of p42/p44 mitogen-activated protein kinase. *J. Biol. Chem.* 273, 13317–13323.
- [20] Le Goff, P., Montano, M.M., Schodin, D.J. and Katzenellenbogen, B.S. (1994) Phosphorylation of the human estrogen receptor. Identification of hormone-regulated sites and examination of their influence on transcriptional activity. *J. Biol. Chem.* 269, 4458–4466.
- [21] Bunone, G., Briand, P.A., Miksicek, R.J. and Picard, D. (1996) Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. *EMBO J.* 15, 2174–2183.
- [22] Chen, D., Riedl, T., Washbrook, E., Pace, P.E., Coombes, R.C., Egly, J.M. and Ali, S. (2000) Activation of estrogen receptor alpha by S118 phosphorylation involves a ligand-dependent interaction with TFIIH and participation of CDK7. *Mol. Cell* 6, 127–137.
- [23] Simoncini, T., Rabkin, E. and Liao, J.K. (2003) Molecular basis of cell membrane estrogen receptor interaction with phosphatidylinositol 3-kinase in endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* 23, 198–203.
- [24] Joel, P.B., Smith, J., Sturgill, T.W., Fisher, T.L., Blenis, J. and Lannigan, D.A. (1998) pp90rsk1 regulates estrogen receptor-mediated transcription through phosphorylation of Ser-167. *Mol. Cell Biol.* 18, 1978–1984.
- [25] Lannigan, D.A. (2003) Estrogen receptor phosphorylation. *Steroids* 68, 1–9.
- [26] Yamashita, H., Nishio, M., Kobayashi, S., Ando, Y., Sugiura, H., Zhang, Z., Hamaguchi, M., Mita, K., Fujii, Y. and Iwase, H. (2005) Phosphorylation of estrogen receptor alpha Ser 167 is predictive of response to endocrine therapy and increases postrelapse survival in metastatic breast cancer. *Breast Cancer Res.* 7, R753–R764.