# Effect of Dietary and Environmental Endocrine Disruptors on Estrogen Metabolic Enzyme Expression

**Ye Lan**

A Thesis Submitted in Partial Fulfillment

of the Requirements for the Degree of

Doctor of Philosophy

in

Biochemistry



July 2009





ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 - 1346

# Thesis committee

**Professor T.T. Kwok (Committee Chairman)**

**Professor L.K. Leung (Thesis supervisor)**

**Professor Franky L. Chan (Committee member)**

**Professor Jennifer M.F. Wan (External examiner)**

# ACKNOWLEGEMENTS

I would like to express my sincere gratitude to my supervisor, Dr. L.K. Leung, for his great support, encouragement and patient guidance during the whole period of my study. I benefit a lot from his guidance and advice on my PhD project. In addition, I would like to thank Dr. T.T. Kwok and his students for their great help on my project. Also, I would like to thank Dr. F.L. Chan for his useful suggestion on how to establish the animal model.

I am grateful to all my labmates working in MMW507, for their friendship, assistance and technical support. Moreover, I would like to thank all my colleagues and friends in the Department of Biochemistry, The Chinese University of Hong Kong, for their kind assistance.

Finally, special thanks are given to my parents, my brother and my boyfriend for their love and encouragement.

# ABSTRACT

Breast cancer is one of the most prevalent female cancers in Hong Kong and western countries. Prolonged exposure to estrogen has been associated with increased risk of breast cancer. Many enzymes are responsible for estrogen metabolism, for instance, aromatase (CYP19) is responsible for biosynthesis; CYP1 family enzymes hydroxylate estrogen; COMT (catechol-O-methyltransferase) inactivates the hydroxyestrogen; and UDP-glucuronosyltransferase 1A1 (UGT1A1) eliminates the estrogen metabolites. In this project, we employed cell and animal models to address estrogen metabolism-related questions under the influence of endocrine disruptors.

TCDD is a prototype compound of a whole class of halogenated aromatic hydrocarbons termed dioxin-like contaminants, which are also known to be endocrine disruptors. Because of their persistence in the environment dioxins are one of the most concerned classes of carcinogens. Humans can be exposed to this pollutant through contaminated food, air, drinking water, etc. We found that pre-ovariectomy administration of TCDD could significantly reduce aromatase expression in the brain but increase the expression in the adipose tissue. Our results suggested that the timing of exposure to the toxicant could determine the estrogenicity of TCDD.

Because of the structural resemblance to the female hormone, phytoestrogen is another important class of endocrine disruptor. In the present project, we evaluated the effects of phytoestrogens isoliquiritigenin (ILN), hesperetin (HES), genistein, (GEN) and naringenin (NAR) on estrogen metabolism and also their effects on MCF-7 tumor growth in ovariectomized nude mice. We found that these phytoestrogens had

differential effect on MCF-7 xenografts. NAR and GEN had totally different responses in the tumor growth. In contrast, ILN and HES only deterred MCF-7 xenograft growth when CYP19 was overexpressed in the graft.

The present project indicated that endocrine disruptors can alter the metabolism of estrogen; however, the significance of this alteration may be specific to tissues' phenotype and the timing of exposure.

# 摘要

在香港和西方國家裏，乳腺癌是女性常見的腫瘤。長期接觸雌激素和乳腺癌的發病率上升有著緊密的聯系。

衆多的酶參與了雌激素的代謝，舉例來講，芳香化酶(CYP19)是雌激素生物合成過程中的關鍵酶；CYP1 家族酶羥基化雌激素；兒茶酚氧位甲基轉移酶(COMT)甲基化羥基化的雌激素；尿苷二磷酸葡萄糖醛酸酶(UGT1A1) 進一步消除雌激素的代謝産物。在此項研究中，我們通過采用細胞和動物的模型來闡述在內分泌幹擾素作用條件下雌激素代謝的有關問題。

鹵化芳香化氫化物被廣泛認為是一種內分泌幹擾素，而二惡英是鹵化芳香化氫化物的一個常見化合物。因為它們在環境中長期留存不易分解，二惡英被認為是一類重要的致癌物。二惡英汙染物主要是通過食用被汙染的食物，空氣以及飲用水等途徑。研究發現，在切卵巢前灌胃二惡英的大鼠中，芳香化酶的表達在腦中降低，相反芳香化酶的表達在脂肪組織中增加。結果表明，二惡英能否發揮其類雌激素的特性和二惡英的作用時間有關。

植物雌激素和雌激素的結構類似，屬於另一類內分泌幹擾素。在此項研究中，我們調查了異甘草素(ILN)，橙皮素(HES) ，染料木素(GEN)和柚皮素(NAR)對雌激素代謝的影響，並進一步研究了它們在小鼠中對乳腺癌的作用。研究發現，這些不同的植物雌激素對 MCF-7 腫瘤有著不同的影響。NAR 和 GEN 對腫瘤有著完全相反的作用。相反 ILN 和 HES 抑制乳腺癌細胞 MCF-7aro 的增殖。

研究表明，內分泌幹擾素能夠影響雌激素的代謝，然而它們能否特異性發揮作用與其作用的組織及其時間相關。

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AhR | Aryl Hydrocarbon Receptor |
| AP-1 | Activator protein-1 |
| Amp | Ampicilin |
| Bcl-2 | B-cell leukemia/lymphoma 2 |
| bFGF | Basic fibroblast growth factor |
| bp | Base pair |
| BSA | Bovine serum albumin |
| cAMP | Cyclic adenosine monophosphate |
| cDNA | Complementary deoxyribonucleic Acid |
| C/EBP | CCAAT/enhancer binding protein |
| COMT | Catechol-O-methyltransferase |
| COX-2 | Cyclooxygenase-2 |
| CRE | cAMP response element |
| CREB | CRE binding protein |
| CYP | Cytochrome P450 |
| DAG | Diacylglycerol |
| dATP | Deoxyadenosyl triphosphate |
| dCTP | Deoxycytosinyl triphosphate |
| dGTP | Deoxyguanosinyl triphosphate |
| DMEM/F12 | Dulbecco's Modification of Eagle's Medium/Ham's F12 |
| DMSO | Dimethyl sulfoside |
| DNA | Deoxynucleic acid |
| dNTP | Deoxyribonucleotide Triphosphate |
| DTT | Dithiothreitol |
| dTTP | Deoxythymidunyl triphosphate |
| E2 | 17β-estradiol |
| E.coli | Escherichea coli |
| EDTA | Ethylenediaminetetraacetate |
| EGF | Epidermal growth factor |
| EGFR | Epigermal growth factor receptor |
| ERBB2 | erythroblastic leukemia viral oncogene homolog 2 |
| ERE | Estrogen response element |
| ERK 1/2 | Extracellular signal-regulated kinase 1/2 |
| ER | Estrogen receptors |
| EtOH | Ethanol |
| FBS | Fetal bovine serum |
| Fos | v-fos FBJ murine osteosarcoma viral oncogene homolog |
| FW | Formula weight |

| | |
|---|---|
| GEN | Genistein |
| HEPES | N-2-hydroxy-ethyl-piperazine-N'-2-Ethane-sulfonic acid |
| HES | Hesperetin |
| HER2 | Human epidermal growth factor receptor 2 |
| IL | Interleukin |
| ILN | Isoliquiritigenin |
| $IP_3$ | Iinositol-1,4,5-triphosphate |
| JNK | c-Jun N-terminal kinase |
| Jun | v-jun avian sarcoma virus 17 oncogene homolog |
| kb | Kilo-base |
| kDa | Kilo-dalton |
| LB | Luria-Broth Medium |
| LBD | Ligand binding domain |
| LPS | lipopolysaccharide |
| MAP | Mitogen-activated protein |
| MAPK | Mitogen-activated protein kinase |
| MBD | Membrane binding domain |
| MEK | Mitogen-activated protein / Extracellular signal-regulated kinase kinase |
| mRNA | Messenger ribonucleic acid |
| MTT | 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide |
| NAR | Naringenin |
| NFAT | Nuclear factor of activated T-cells |
| NFκB | Nuclear factor kappa B |
| NF-IL6 | Nuclear factor interleukin-6 |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| $PIP_2$ | phosphoinositol-4,5-bisphoaphate |
| PI3 kinase | phosphatidylinositol 3-kinase |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| RNA | Ribonucleic acid |
| RNase | Ribonuclease |
| rpm | Revolutions per minute |
| RPMI 1640 Medium | Rosewell Park Memorial Institute Tissue Culture Medium 1640 |
| RT-PCR | Reverse transcription-polymerase chain reaction |
| SD | Female Sprague Dawley |
| SDS | Sodium dodecyl sulfate |
| TAE | Tris-acetate-EDTA |
| TCDD | 2,3,7,8-tetrachlorodibenzo-para-dioxin |
| TGFα | Transforming growth factor alpha |

| | |
|---|---|
| TGFβ-1 | Transforming growth factor beta-1 |
| Tris | Trizma base |
| UGT1A1 | UDP-glucuronosyltransferase 1A1 |
| XRE | Xenobiotic Response Element |

# CHAPTER 1
# GENERAL INTRODUCTION

Breast cancer is the most dominant type of cancer affecting women and the second leading cause of cancer-related deaths in women of the western countries (Nkondjock and Ghadirian 2005). One hundred and eighty four thousand new cases, contributing to 26% of all cancer cases, are diagnosed in the United States in 2008 (American Cancer Society, 2009). In Hong Kong the breast cancer incidence and mortality rate in female were 72 and 12.9 per 100,000 in 2006 (Hong Kong Cancer Registry, 2008).

Breast cancer is found more frequently in postmenopausal women, and the hormone-sensitive tumor incidence is higher (60%) than that in premenopausal women (Brodie *et al.* 1997).Numerous risk factors for breast cancer have been identified, including gender (women more than male), family history (heritability/loss of function of susceptibility genes BRCA-1 and BRCA-2), age, steroid-hormonal status (including age of first/last menarche and first pregnancy, breast feeding) and post-menopausal obesity (Nkondjock and Ghadirian 2005).

## 1.1 Estrogen and Breast Cancer

Estrogen is an essential hormone for the development of reproductive system in female (Sebastian and Bulun 2001). Estrogen plays a critical role in the development and progression of breast cancer. Approximately 60% of pre-menopausal and 75% of postmenopausal breast cancer patients have estrogen-dependent carcinomas (Zhou *et*

*al.* 1996). The ovary ceases to produce estrogen in postmenopausal women and their plasma levels drop by 80–90% compared to those of premenopausal women (Lonning *et al.* 1997; Shields-Botella *et al.* 2005). Despite the fact that the circulating level of estrogen is relatively low in postmenopausal women, they are more liable to get breast cancer than premenopausal women (Jelovac *et al.* 2004). Production of estrogens in peripheral tissue proximate to the breast increases and the estrogen levels in the breast are the same in the postmenopausal women as in premenopausal women (Jelovac *et al.* 2004). Emerging evidence indicates that estrogen produced in situ in the breast of postmenopausal women plays an important role in tumor proliferation and progression (Grube *et al.* 2001). The locally produced estrogen binds to the estrogen receptor and induces the expression of growth factors which are responsible for cell proliferation (Chen 2002; Chen *et al.* 2002).

## 1.2 Estrogen Synthesis and Metabolism

Estrogen can be synthesized from cholesterol in several steps. In postmenopausal women, the primary source of estrogens is from the peripheral tissues such as breast stromal cells, breast tumor tissues, muscles, bones and adipose tissues. Circulating steroids such as androstenedione, testosterone, and estrone sulfate are the major precursors for estrogen synthesis. Aromatase is the key enzyme which catalyzes estrogen synthesis and many enzymes are responsible for the hormone's metabolism. Cytochrome p450 I family enzymes are responsible for the hydroxylation of estrogen (Tsuchiya *et al.* 2005). CYP1A1 is the enzyme that

hydroxylates estrogen at the position of C-2, and its expression is inducible by PAH through the aryl hydrocarbon receptor (AhR) activation pathway (Spink *et al.* 1998; Nebert *et al.* 2004). Polymorphisms with augmented CYP1A1 inducibility in African-Americans (Taioli *et al.* 1995) and Chinese (Huang *et al.* 1999) are associated with increased breast cancer risk. However, this association fails to hold in Indian women (Singh *et al.* 2007) or Korean women (Shin *et al.* 2007). CYP1B1 hydroxylates estrogen at the C-4 position to form 4-hydroxyestrogen. The formation of 4-hydroxyestradiol generates free radicals from reductive-oxidative cycling with the corresponding semiquinone and quinone forms, which cause DNA damage. Moreover, the hydroxylated metabolite of estrogen retains all the estrogenic properties and can be more damaging with regard to its genotoxicity than the parent compound (Liehr 2000; Roos and Bolt 2005). Moreover, polymorphisms of *CYP1B1* are associated with increased risk of breast cancer (De Vivo *et al.* 2002; Zimarina *et al.* 2004). Moreover, other hydroxylated (2-, 4-, 6α-, 6β-,7α-, 12β-, 15α-, 15β-, 16α-, and 16β- hydroxylated) metabolites of estrogen have been detected. UDP-glucuronosyltransferase 1A1 (UGT1A1) catalyzes the formation of estrogen-glucuronide (Shatalova *et al.* 2006). A low expression of UGT1A1 may increase the circulating estrogen. An increased association was observed between UGT1A1 expression and breast cancer risk in Africa Americans and Russian women (Guillemette *et al.* 2000; Shatalova *et al.* 2006). Yueh *et al.* (2001) have suggested that early down-regulation of the UGTs in premalignant and malignant tumor tissues may be a prerequisite of cellular carcinogenesis. We have demonstrated that an

elevated expression level of UGT1A1 in MCF-7 cells was associated with decreased cell proliferation (Leung *et al.* 2007). Catechol-O-methyltransferase (COMT) inactivates 2-hydroxy-estradiol and 4-hydroxy-estradiol by methylation. The methylated catechol estrogens will be further glucuronidated and eliminated by UGT (Hamajima *et al.* 2001). Recent studies also found that induced activity of COMT increases risk of breast carcinogenesis (Creveling 2003).

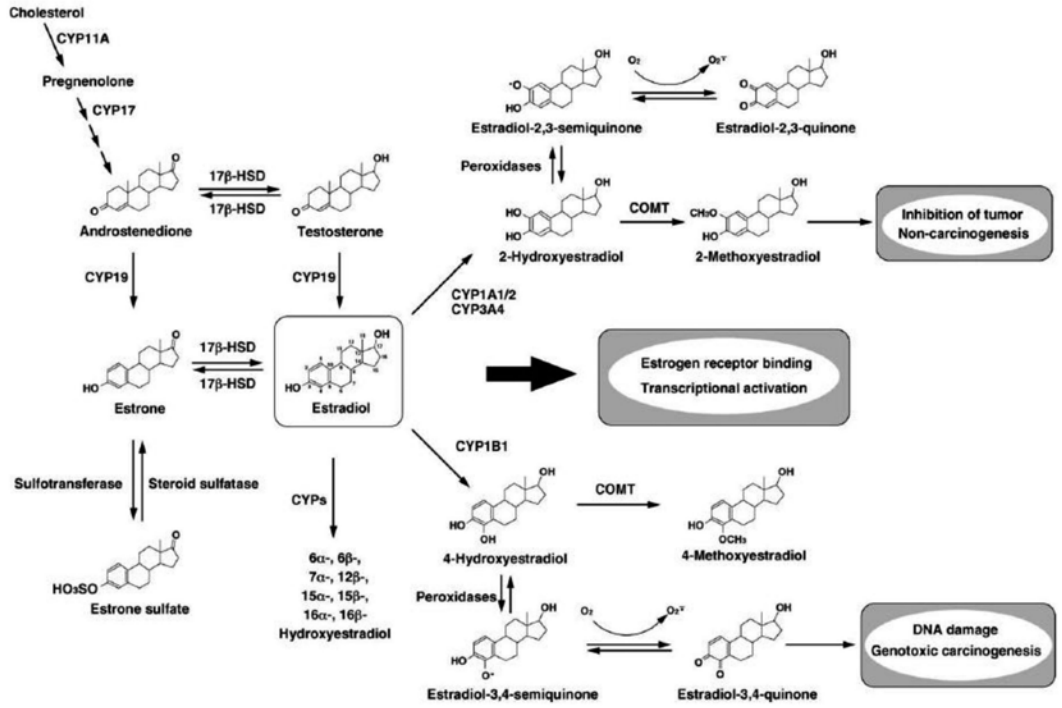



**Fig.1.1.** Cytochrome P450-mediated metabolic pathways of estradiol. Estradiol is synthesized from cholesterol by several steps and CYP19 is the key enzyme for the estrogen synthesis. Estradiol is metabolized to 2- and 4-hydroxyestradiol. These catechol metabolites can be subsequently methylated by COMT. The formation of 4-hydroxyestradiol generates free radicals such as superoxide and the reactive semiquinone/quinone intermediates, which are demonstrated to be carcinogenic in animals. Adopted from (Tsuchiya *et al.* 2005).

## 1.3 Aromatase and Tissue Specific Promoter for Aromatase Expression

Aromatase is the critical enzyme synthesizing estrogens by converting C19 androgens to aromatic C18 estrogenic steroids (Zhou *et al.* 1996; Kao *et al.* 1998; Sebastian and Bulun 2001; Kijima *et al.* 2006). In the postmenopausal period,

testosterone produced in the adrenal cortex, is converted into estrogens in adipose, muscle and connective tissue through the action of CYP19 (Chen 1998). Abnormal expression of aromatase in breast cancer cells may play a dominant role in tumor proliferation and development (James *et al.* 1987; Miller *et al.* 2001). The in situ estrogen synthesis in postmenopausal women is thought to be more critical in breast tumor promotion than circulating estrogen (Yue *et al.* 1994; Kijima *et al.* 2006). In postmenopausal patients, concentrations of estrogen in breast tumors were several-fold higher than those in plasma (Pasqualini *et al.* 1996) and a highly significant correlation between aromatase activity and breast cancer incidence has been found (Chen *et al.* 1999). Further studies have shown that the expression level of aromatase in human breast cancer tissue is higher than that in normal breast tissue, as measured by enzyme activity assay, immunocytochemistry, and reverse transcription-polymerase chain reactions (RT-PCR) analysis (Esteban *et al.* 1992; Zhou *et al.* 1996; Chen *et al.* 1999; Miller *et al.* 2001). In addition, tumor aromatase stimulates breast cancer growth in both an autocrine and a paracrine manner (Sun *et al.* 1997).

Aromatase is encoded by a single copy of *CYP19* gene, which is localized at chromosome 15q21.2 by in situ hybridization studies (Chen *et al.* 1988; Simpson *et al.* 1994). It spans about 123 kb and composes of 10 exons. Only the 30-kb 3'-region encodes aromatase, whereas a large 93-kb 5'-flanking region serves as the regulatory unit of the gene (Sebastian and Bulun 2001). A complex mechanism is involved in the control of human aromatase expression. In human, the expression of *CYP19* is

regulated by alternative splicing (Mahendroo *et al.* 1993; Simpson *et al.* 1994; Harada *et al.* 2003). As shown in **Fig.1.2**, the coding region comprises nine translated exons and a number of untranslated first exons lie upstream of the coding region. Each exon I is flanked by its own unique promoter region and is spliced onto a common splice junction immediately upstream of the start of translation. Hence, the opening reading frame of each transcript (exon II to X), as well as the CYP19 protein, is identical irrespective of the promoter used and site of expression.

Recruitment of this most distal promoter may have an evolutionary impact as only humans are unique to acquire and maintain extraordinarily high levels of aromatase expression in placenta (Sebastian and Bulun 2001).Up to now, there are ten distinct tissue-specific promoters: PI.1 (placenta, major), PI.2 (placenta, minor), PI.3 (adipose/ breast cancer), PI.4 (skin and adipose), PI.5 (fetal tissue), PI.6 (bone), PI.7 (endothelial), PI.f (brain), PII (ovary/breast cancer/endometriosis) and P2a (placenta, minor) (Shozu et al., 1998; Sebastian et al., 2001; Sebastian et al., 2002) (**Fig.1.2**). In breast cancer, exons I.3 and II are the most frequently used, suggesting the promoter I.3 and II are the major promoters directing aromatase expression in the malignant and surrounding tissue (Zhou *et al.* 1996). In normal breast adipose stromal cells and fibroblasts, aromatase expression is driven by promoter I.4, a glucocorticoid-stimulated promoter, and that the action of promoters I.3 and II is suppressed by the silencer element (S1). S1 is a negative regulatory element that is situated between promoters I.3 and II and inhibit the function of these promoters (Zhou and Chen 1998). When the cAMP increases the aromatase promoter is

switched from I.4 in normal tissue to promoters I.3 and II, cAMP-stimulated promoters in cancer cells and surrounding adipose stromal cells (Chen *et al.* 1999). A new cAMP-responsive element (CREaro) upstream from promoter I.3 has been identified recently by DNA deletion and mutational analyses. The underlying mechanism of promoter usage switch may be that the positive regulatory CREaro can overcome the action of the S1 on the function of promoter I.3 in the presence of cAMP (Chen *et al.* 1999).

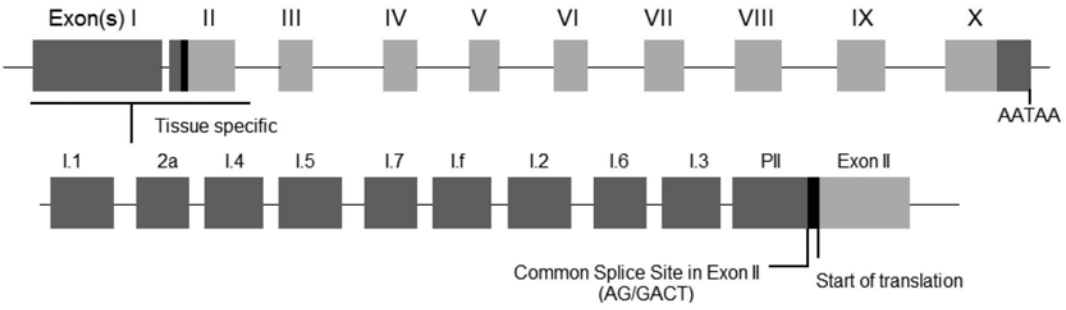



**Fig.1.2.** Schematic representation of aromatase gene. A *CYP19* overview shows the exons and multiple sub regions of exon I. Dark gray shading, untranslated region; Light gray shading, translated region. Modified from (Sebastian and Bulun 2001; Ellem *et al.* 2004).

## 1.4 Signaling Pathway

MAPK cascades are evolutionary conserved signal transduction modules that are used in a wide variety of biological responses. In vertebrates, multiple isoforms of MAPK have been identified and categorized into three subfamilies, i.e. the extracellular signal-regulated kinases (ERKs), p38mapk, and the Jun N-terminal

kinases (JNKs) or stress-activated protein kinases. The 'classical' ERKs, ERK2 or p42mapk and ERK1 or p44mapk, are positioned downstream of Raf-1 and MEK1, and together comprise an orderly signaling cascade in response to a variety of extracellular stimuli including growth factors (Davis 1993; Garrington and Johnson 1999; Torres 2003). Generally, JNK and p38 kinase pathways may serve as transducers for injury or stress responses, whereas ERK pathways are more specialized for growth and mitogenic factor stimulation (Garrington and Johnson 1999).

The MAPK signaling pathways have been implicated in control of different and even opposite cellular responses including proliferation, differentiation and cell death. Such actions are elicited at least in part through translocation of activated MAPK into the nucleus (Chen *et al.* 2003), where it phosphorylates and thereby activates nuclear transcription factors, including Elk-1 (Gille *et al.* 1992), Sap1 (Dalton and Treisman 1992), c-Jun (Rozek and Pfeifer 1993) and ATF2 (Gille *et al.* 1992). These transcription factors stimulate the expression of the immediate-early response oncogenes, i.e. c-fos (Gille *et al.* 1992); and c-jun (Rozek and Pfeifer 1993). Fos and Jun proteins are constituents of the activator protein-1 (AP-1) transcription factors that in turn regulate the transcription of numerous genes possessing promoter AP-1 binding sites (Karin and Shaulian 2001; Shaulian and Karin 2002). cAMP-dependent protein kinase, which is also known as protein kinase A (PKA), is allosterically activated by cAMP. Protein kinase C (PKC) is activated by elevated concentration of calcium. Moreover, some of the diacylglycerol-dependent isoforms of PKC, i.e. α

(Kolch *et al.* 1993; Cai *et al.* 1997), $\beta_1$ and ε (Cai *et al.* 1997), can activate Raf-1 by direct phosphorylation of serine residues in the regulatory as well as the kinase domain (Kolch *et al.* 1993). Both PKA and PKC can phosphorylate Ser or Thr residues of specific target protein and alters its catalytic activities (Michael *et al.* 1995).

**Fig1.3** illustrates a simplified scheme of MAPK signaling pathway. The stimulus at the cell membrane activates the Ras protein, then Ras would directly couple to Raf and phosphorylates MEK1/2. These two proteins act as dual specificity kinases and directly phosphorylate the TEY motif in ERK1/2 kinases. Similar cascades have been observed in JNK and p38 kinase pathways (Garrington and Johnson 1999).

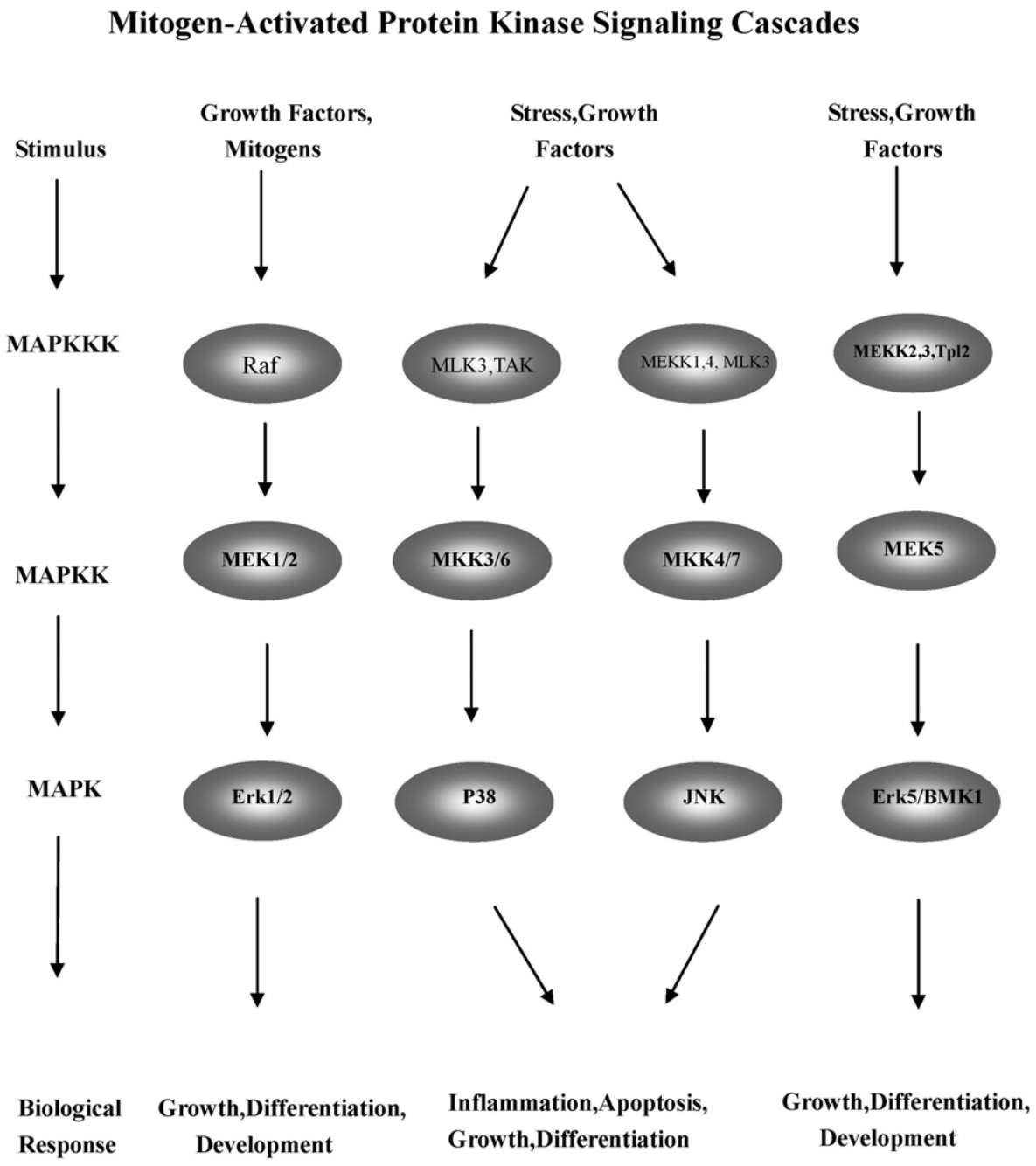



**Fig.1.3.** A simplified scheme illustrates MAPKs signaling pathway (Modified from Cell Signaling Technology).

## 1.5 Nuclear Receptors and Aromatase Promoter Regulation

*CYP19* is a member of the cytochrome P450 gene superfamily, and is one of several eukaryotic genes regulated by tissue-specific promoters. Because the promoter that drives the expression in breast cancer tissues is different from that in normal breast, many studies have designed to address the mechanisms.

Members of the nuclear receptor superfamily are involved in the regulation. Nuclear receptors are transcription factors, which include ligand-activated nuclear hormone receptors (*e.g.* estrogen receptor) and orphan receptors with unknown ligands. All nuclear receptors have a conserved DNA binding domain and can bind to related DNA sequences.

Simpson's research group has focused their study on aromatase expression in adipose fibroblasts and primary cultures of stromal cells isolated from human breast adipose tissue. They have found that the liver receptor homologue-1 (LRH-1) is a preadipocyte-specific orphan nuclear receptor that can increase aromatase promoter II activity (Clyne *et al.* 2002; Clyne *et al.* 2004). In normal breast tissue the expression of aromatase is under the control of the promoter region PI.4, regulated by class I cytokines such as IL-6, IL-11 and Tumor Necrosis Factor-α (TNF-α) with the permissive role of glucocorticoids (GREs). A promoter switch from PI.4 to PI.3 and PII is noticeably found in breast tumor tissue and linked to elevated CYP19 expression (Chen 1998). These promoters are under the control of a cAMP responsive element (CRE) (Michael *et al.* 1995; Sofi *et al.* 2003). Researchers have found that the over-expression of aromatase in adipose tissue surrounding breast

tumors could be caused by increases in expression and transcriptional activity of cAMP-respond element binding protein (CREB) (Michael *et al.* 1995; Sofi *et al.* 2003). AP-1 is also reported to regulate Promoter II activity of the human CYP19 gene in the ovary and malignant breast cells (Vallejo *et al.* 1995; Ghosh *et al.* 2005). It can be a combination of homodimers or heterodimers of the Jun and Fos subfamilies that affects aspects in cell proliferation and differentiation (Karin and Shaulian 2001; Bourguiba *et al.* 2003; Ghosh *et al.* 2005). Mamma-lian Jun proteins include c-Jun, JunB, and JunD; Fos proteins include c-Fos, FosB, Fra1, and Fra2. Fos family and Jun family proteins interact and form hetero Fos-Jun or homo Jun-Jun dimmer, and bind to specific sequences on DNA. In addition, CCAAT/Enhancer Binding Protein Protein (C/EBP) can also control the Promoter II activity in malignant breast epithelial cells (Michael *et al.* 1995; Zhou *et al.* 2001; Sofi *et al.* 2003). Locations and sequences of transcription factor binding sites are shown in **Fig1.4A** and **Fig1.4B**, respectively.

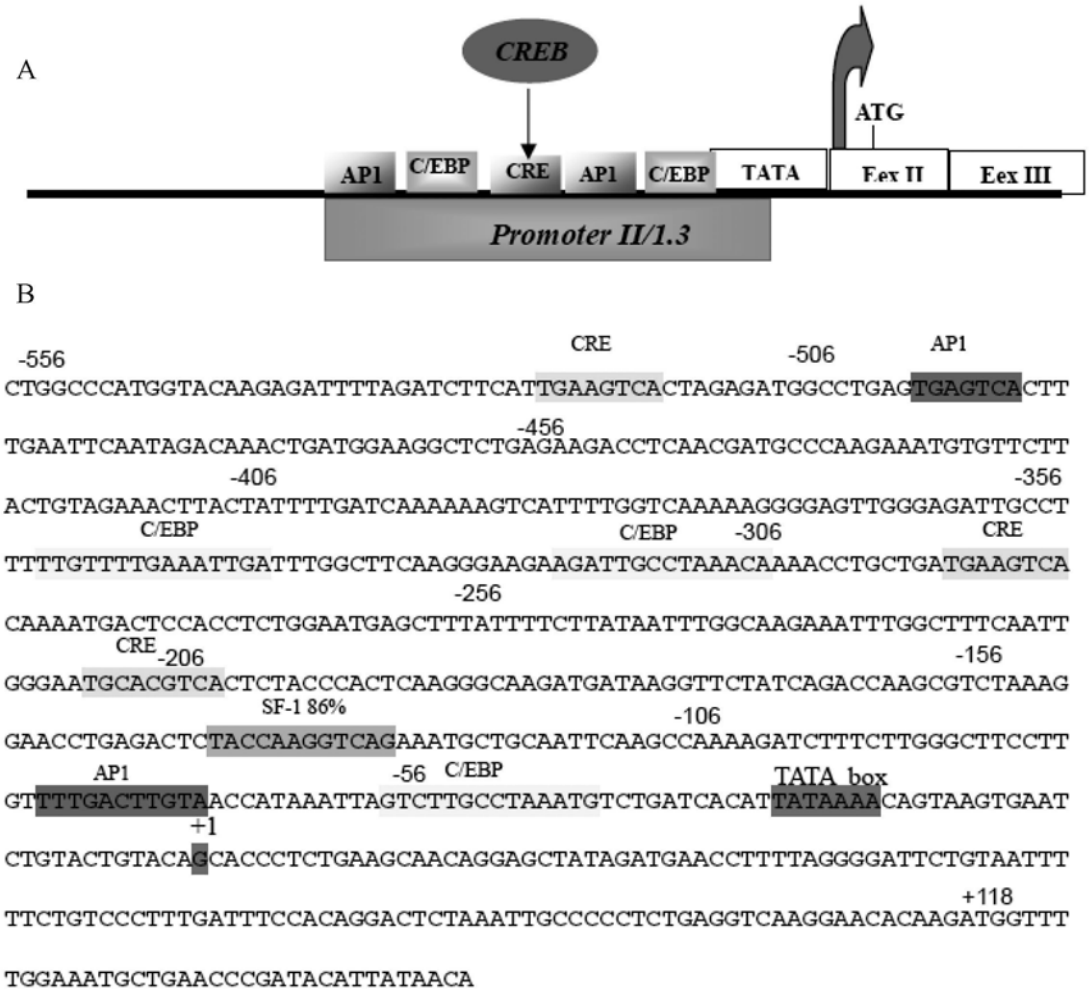



**Fig.1.4.**Schematic representation of parts of transcription binding sites in promoter II of *CYP19* (**A**). Transcription binding sites of promoter 1.3 and II of *CYP19* gene. Some of the major regulatory elements are shown in (**B)** (modified from Zhou *et al*. 2002).

## 1.6 Cell Cycle

Loss of cell growth control is a hallmark of cancer. Cell proliferation control in the normal mammary gland can be regulated by estrogen, which involves complex interactions with other hormones, growth factors and cytokines and ultimately converges on activation of proto-oncogenes (Butt *et al.* 2008). Estrogens have been reported to induce breast cancer cell proliferation via stimulating G(1)/S transition through cyclin D1 and Cdks (Foster *et al.* 2001). The cell cycle is a series of events leading to cell replication. S and M phases are two important processes in cell cycle. Chromosomes are replicated in S phase, and replicated material is segregated into two daughter nuclei in M phase (**Fig.1.5**) (Nurse, 1994; Nasmyth, 1993, 1996). As shown in **Fig.1.5**, the eukaryotic cell cycle is controlled by different cyclin-dependent kinases (CDKs)/cyclin complexes that coordinate with exogenous growth stimulators (hormones, growth factors, signals) at several key checkpoints (Okayama 1994; Grana and Reddy 1995). CDKs are a well-conserved family of serine/threonine protein kinases and are important in the initiation and coordinating the cell cycle (Eisenbrand *et al.* 2004). CDKs needed to be activated by cyclins. Up to now, 9 CDKs and 16 cyclins have been identified in mammalian cells. Cyclin D is a regulatory protein involved in the G1 phase of the cell cycle and its expression is always increased in many cancers (Hiyama *et al.* 1997). The transition from $G_0$ phase to G phase in eukaryotic cells is primarily mediated by cyclin D/CDK4- and cyclin D/CDK6-complexes (Eisenbrand *et al.* 2004). CDK2 binds to cyclin E and later with cyclin A to initiate S-phase progression. Estrogens have been reported to induce

breast cancer cell proliferation via stimulating G(1)/S transition associated with increased expression of cyclin D1 and CDKs (Foster *et al.* 2001). The CDK/cyclin complexes are inhibited by a group of small proteins, known as CDK inhibitors. There are two families of CDK inhibitors: the CIP/KIP-family and the INK4-family (Eisenbrand *et al.* 2004). $p21^{CIP/WAF1}$, $p27^{KIP1}$, $p57^{KIP2}$ belongs to the CIP/KIP-family which inhibit the activity of CDK2, CDK4 and CDK6/cyclin complexes. $p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$, $p19^{INK4d/ARF}$ belongs to INK4-family, which exclusively inhibit CDK4 and CDK6/cyclin complexes (Matsuoka *et al.* 1998; Sherr and Roberts 1999).

Previous studies demonstrated that over-expression cyclin D1 and E1 in mammary epithelial cells induces mammary carcinoma in mice (Butt *et al.* 2008). Furthermore, the expression of cyclin D1, E1 and E2 is always increased in primary breast cancer. Elevated expression of the cyclins is often associated with a more aggressive disease phenotype and an adverse patient outcome. Thus, abnormal cell cycle regulation is likely to be directly involved in the development and progression of breast cancer.

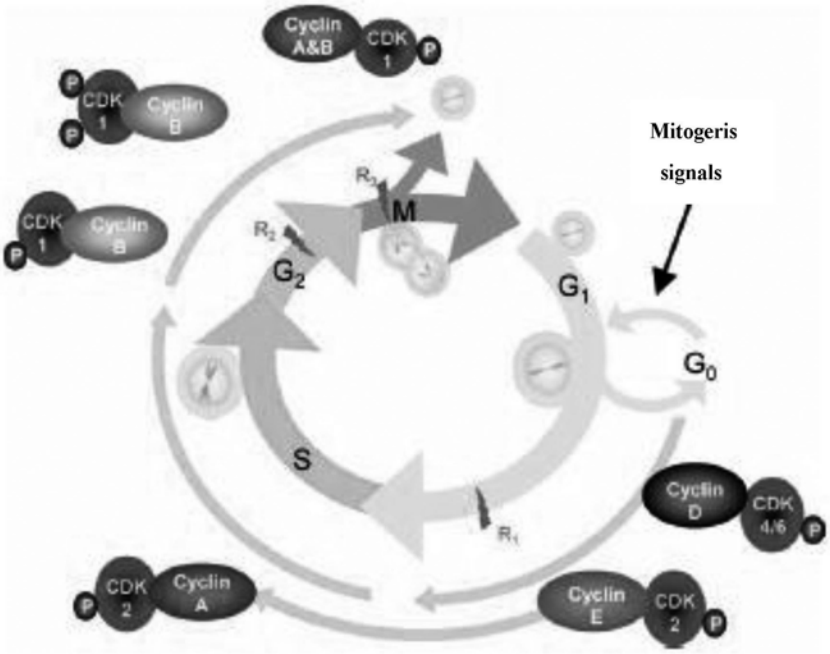



**Fig.1.5.** Cell cycle regulation Adopted from (Eisenbrand *et al.* 2004).

## 1.7 Cell Apoptosis

The blockage of G1/S transition could result in cell apoptosis (Mantena *et al.* 2006; Choi 2007). Apoptosis is an essential physiological process important in controlling cell number and proliferation. Cell survival is determined by the complex interplay between proapoptotic and antiapoptotic Bcl-2 family proteins. Increased expression of antiapoptotic proteins commonly occurs in many cancers and is associated with disease maintenance and progression (Dai and Grant 2007).

The intrinsic apoptotic pathway is characterized by the release of cytochrome c, which activates caspases (Hector and Prehn 2009). This process is controlled by the Bcl-2 family of proteins. Bcl-2 family consists of anti-apoptotic and pro-apoptotic

members (Tsujimoto 1998). Bcl-2 and Bcl-xL belong to the antiapoptotic Bcl-2 family proteins while Bax and Bak are the pro-apoptotic members. Overexpression of Bcl-xL has been demonstrated to induce cell proliferation (Kim 2005). In contrast, activation of Bax and Bak induces the release of cytochrome c from the mitochondria, which triggers the activation of caspases as shown in **Fig.1.6** (van Delft and Huang 2006). Caspases are a family of cysteine proteases, which can be either the initiator or effector caspases. The release of cytochrome c firstly activates the initiator caspase 9, which goes on to activate the effector caspases 3 and 7 (Tsujimoto 1998; Harada and Grant 2003; van Delft and Huang 2006).

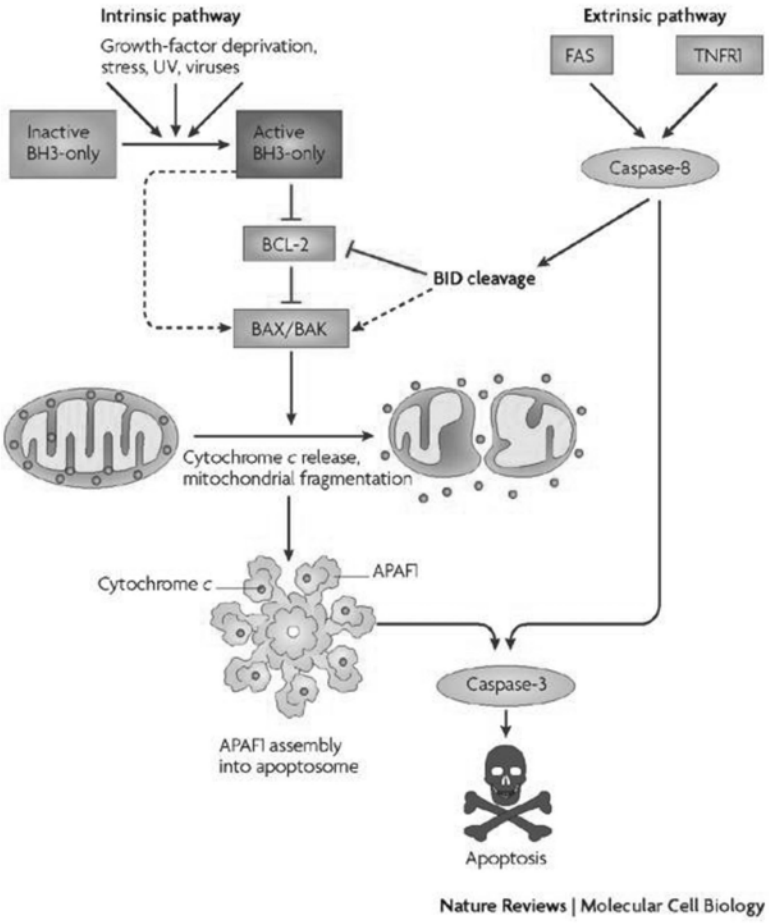



**Fig.1.6.** Cell apoptosis Adopted from (Youle and Strasser 2008).

## 1.8 Treatment of Breast Cancer

Dependent on the stage and biological characteristics of breast cancer, most breast cancer patients will have some type of surgery (Reed *et al.* 2009). Surgery is often combined with other treatments such as radiation therapy, chemotherapy, hormone therapy. Radiation therapy is used to destroy cancer cells remaining in the breast, chest wall, or underarm area after surgery (Hanagiri *et al.* 2008; Recht 2008). Chemotherapy refers to the use of chemotherapeutic drugs after the tumor has been removed. Both radiation and chemotherapy have severe side effects. Hormone therapy is to reduce the estrogen which has been demonstrated to promote cell proliferation in breast cancer. Two strategies can be employed to reduce the effects of estrogen on tumor growth. One is the use of antiestrogens, and the other is inhibiting of estrogen synthesis. The antiestrogen tamoxifen has been a primary treatment for breast cancer since the 1970s and it has been very effective in treating estrogen receptor positive breast cancer (Shen *et al.* 2008). However, tamoxifen is a partial agonist as well as an antagonist. It can cause endometrial hyperplasia and occasionally endometrial cancer. It also increases risk of stroke because of its estrogenic effects (Hernandez *et al.* 2008; Lindahl *et al.* 2008). Moreover, the two approaches have been compared in recent clinical trials and the result suggests that aromatase inhibition is more effective than tamoxifen as first-line therapy for postmenopausal patients with hormone-responsive breast cancer and for patients with early breast cancer (Jelovac *et al.* 2004). Two classes of aromatase inhibitors, steroidal (formestane, exemestane) and nonsteroidal (anastrozole, letrozole), have

been developed for such purpose (Ikeda *et al.* 2008; Lonning and Geisler 2008).

## 1.9 Endocrine Disruptors and Breast Cancer

Endocrine disruptors are a range of synthetic and natural substances, which possess both pro- and antiagonistic properties to some hormone receptors. In recent years evidences have shown that exposure to endocrine disruptors may result in breast cancer and endometriosis (Foster 2008). Xenoestrogens and phytoestrogens are the two major endocrine disruptors displaying estrogenic activity. Epidemiological reports indicate that the xenoestrogens may be responsible for some cases of breast carcinogenisis. The 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is a prototype compound of a whole class of halogenated aromatic hydrocarbons (Knerr and Schrenk 2006). TCDD and dioxin-like PCDDs and PCDFs are produced as unwanted byproducts of many industrial and combustion processes. They are widely distributed in food, human tissue, breast milk, and environmental samples (Rier and Foster 2002). They would induce or block gene expressions like cytochrome P-450 and genes involved in cellular growth, differentiation, and inflammation. Because of their persistence in the environment dioxins are one of the most concerned classes of carcinogens. A positive association of TCDD exposure and breast cancer risk has been demonstrated by several research groups (Brown *et al.* 1998; Revich *et al.* 2001; Warner *et al.* 2002). Some animal studies have also demonstrated that TCDD treatment increases the number of 7,12- dimethylbenz[a]anthracene (DMBA) or methyl- nitrosourea (MNU)-induced mammary tumors in rats (Brown *et al.* 1998; Desaulniers *et al.* 2001).

Phytoestrogens are another class of endocrine disruptors. They are plant-derived, non-steroidal compounds with structural similarity to estrogen. Fruits, vegetables, tea leaves, soybeans and herbs are major sources of phytoestrogens. There are three main classes of phytoestrogens: flavonoids, coumestans, and lignans. Flavonoids can be classified into different subgroups like flavanols, flavanones, flavones, isoflavones, anthocyanins and chalcones (Miranda *et al.* 1999; Whitehead and Lacey 2003). Women in Asian countries have a lower incidence of breast cancer than those in the Western countries, and Asian descendants who migrated to the US would lose this advantage (Ziegler *et al.* 1993). The difference in soyabean consumption has been claimed to be a major contributing factor. Genistein is a soy isoflavone that has been the focus of many studies regarding its health benefits. In cell culture studies, genistein in pharmacological doses has been found to inhibit growth of many cell types including mammary cancer cells (Kuo, 1997). Suppressive effects of genistein on MCF-7 tumor growth have been reported (Shao *et al.* 1998). Genistein has also been reported to induce cell apoptosis in MCF-7 and T47D cells (Yu *et al.* 2003). Moreover, the isoflavone shares some common structure with the hormone estrogen. It is suggested that genistein may act as a selective estrogen receptor modulator (SERM) (Kuiper *et al.* 1998).

In recently years, phytoestrogens have been found to have anti-aromatase, antiestrogenic or antiproliferative actions. They may function as antiestrogens or weak estrogen receptor agonists. Some phytoestrogens bind to the active site of aromatase in which their rings-A and -C mimic rings-D and -C of the substrate

androgen (Graham-Lorence *et al.* 1995). Thus, these phytoestrogens act similar to pharmaceutical aromatase inhibitors such as fadrozole and could therefore have a preventive role in breast cancer therapy. However, they may also activate the estrogen receptor (ER) and cause cancer cell proliferation (Kao *et al.* 1998; Kuiper *et al.* 1998).

## 1.10 Aim of My Study

Breast cancer is one of the most prevalent female cancers in Western countries and in Hong Kong. One in every 24 Hong Kong women will develop breast cancer in their lifetime. Patients who are subjected to excessive estrogen exposure have increased risk for breast cancer development.

Exposure to dioxins may lead to developmental and reproductive abnormalities, and cancer (Birnbaum and Tuomisto 2000; Steenland *et al.*, 2004). However, its mechanism of toxicity has not been clearly established. The association between 2,3,7,8-tetrachlorodibenzo-paradioxin (TCDD) exposure and breast cancer risk has been inconclusive in epidemiological studies (Manz *et al.* 1991; Safe and Zacharewski 1997; Warner *et al.* 2002).

As stipulated in the Dietary Supplement Health and Education Act of the U.S., a dietary supplement or nutraceutical is a food ingredient which must be taken orally and is meant to supplement the diet for disease prevention. Since their acute toxicity has been reasonably established, food phytochemicals have attracted much attention with regard to their potential in health application. Perimenopausal symptoms have troubled many middle-aged women, and hormone replacement therapy (HRT) has been a standard treatment. The side effects of HRT have been well documented, and people have looked into alternative treatment for menopausal symptoms. Extracts from botanicals, such as soybeans, red clover, black cohosh and licorice, etc., have been marketed as dietary supplements for such symptoms. Some phytochemicals that can be isolated from these botanicals are structurally similar to the female hormone

estrogen and have been given the name phytoestrogens. Purified phytoestrogens are now available in the market as a natural alternative to HRT, therefore the possible beneficial as well adverse effects on human health should be considered and evaluated.

The objective of my study was to investigate effects of TCDD and phytoestrogens on estrogen metabolic enzyme expression and the inhibitory or potentiation mechanisms on the growth of ER-positive breast cells *in vitro* and *in vivo*. Our hypothesis is that these compounds can promote or disrupt the carcinogenic process by altering the metabolism of estrogen.

# CHAPTER 2
# MATERIALS AND METHODS

## 2.1 Chemicals and Materials

### 2.1.1 Chemicals

Hesperetin and isoliquiritigenin was obtained from Indofine Chemical Co., Inc., Hillsborough, NJ, USA. It was dissolved in dimethylsulphoxide (DMSO) at a concentration of 100 mM as the stock. The mitogen-activated protein (MAP) kinase inhibitor U0126, p38 MAP kinase inhibitor SB203580, the protein kinase A (PKA) inhibitor myristoylated 14-22 amide and the protein kinase C (PKC) inhibitor bisindolylmaleimide I were obtained from Calbiochem (San Diego, CA). All restriction enzymes were purchased from New England Biolabs (Ipswich, MA, USA). All primers were synthesized by Invitrogen (Grand Island, NY, USA). ICI 182 780 was a gift from Dr. Y. Huang (Physiology Department, the Chinese University of Hong Kong, HKSAR, China). All other chemicals, if not stated, were acquired from Sigma Chemical.

### 2.1.2 Plasmids

Mammalian expression vector pcDNA3.1+ was a gift from Dr. Y.Y. Ho (Department of Biochemistry, The Chinese University of Hong Kong, HKSAR,China). pcDNA3.1-c-Jun has been prepared in our lab. Firefly luciferase reporter plasmid pTA-luc was purchased from Clontech (Mountain View, CA, USA). Renilla luciferase control plasmid pRL was purchased from Promega Crop (Madison, WI, USA).

## 2.2 Cell Culture

The breast cancer cell line MCF-7 cells and liver cancer cell line HepG2 cells were purchased from A.T.C.C., Rockville, MD. MCF-7 cells stably transfected with human CYP19 (MCF-7aro) were a gift from Dr S Chen (Division of Immunology, Beckman Research Institute of the City of Hope, Duarte, U.S.A.). The stably transfected MCF-7 cells and HepG2 cells were maintained in RPMI medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Invitrogen Life Technology, Rockville, MD) and the selection antibiotic 500 μg/ml G418 (USB, Cleveland, OH, USA). Cells incubated at 37ºC, 5 % carbon dioxide and routinely sub-cultured when reaching 80% of confluency. HES, ILN, NAR and GEN was administered in the solvent vehicle dimethyl sulphoxide (DMSO), and the concentration was limited to 0.1% v/v. Cell density was seeded uniformly at $5 \times 10^2$ cells/$mm^2$ in all experiments.

## 2.3 Aromatase Activity Assay

### 2.3.1 'In-cell' Aromatase Assays

These assays were performed as previously described. In brief, MCF-7aro or HepG2 cells were seeded and allowed 1 day for attachment. Assays were started by replacing the culture medium with serum-free medium containing [1β-$^3$H(N)]-androst-4-ene-3,17-dione (NET-926, PerkinElmer Life and Analytical Sciences, Boston MA, USA) and testing compound. The final concentration of androstenedione was controlled at 25nM, and the reaction was incubated at 37ºC for 1 hr. An aliquot of the medium was then mixed with equal volume of chloroform,

followed by a 10,000 × g centrifugation at 4ºC for 10 min. The aqueous phase was removed into a new tube containing 500µl of 5% activated charcoal suspension. After 30 min incubation, an aliquot of the supernatant fraction was taken out for scintillation counting. The protein content of the cells, on the other hand, was determined by using a BCA kit (Sigma Chemicals) after dissolving the cells in 0.5 mol/l NaOH.

For the enzyme inhibition assays performed on recombinant protein, 2 pmol Supersomes® was incubated with testing compound and substrate-containing assay buffer (25nM [1β-3H(N)]-androst-4-ene-3, 17-dione, 3.3mM $MgCl_2$, 100mM $KH_2PO4$ (pH7.4)). The reaction was initiated by the addition of 1.3mM NADPH and incubated at 37°C for 15 min.

### 2.3.2 Aromatase Activity Assay in Tissues

The assay was modified as previously described (Cos et al., 2006). In brief, frozen tissues were homogenized on ice in 50mM potassium phosphate buffer (pH 7.4) with 250mM sucrose, 10mM dithiothreitol and 3mM magnesium chloride. The homogenates were initially centrifuged for 10 min at 850 g. The supernatants were collected and centrifuged at 100,000 g for 60 min to obtain the microsomes. The isolated microsomes were resuspended in the homogenizing buffer and stored at -80°C until assayed.

The assay contained a NADPH-generating system (5mM NADP, 50mM glucose-6-phosphate, 2 U/ml glucose-6-phosphate dehydrogenase) and substrate-containing assay buffer (25nM-[1β-$^3$H(N)]androst-4-ene-3, 17-dione (NEN Life

Science Products, Boston, MA), 3.3mM-MgCl2, 100mM-$KH_2PO4$ (pH 7.4)). The reaction was incubated at 37°C for 24 h. An aliquot of the supernatant was then mixed with an equal volume of chloroform, followed by a 10,000 g centrifugation at 4°C for 10 min. The aqueous phase was removed into a new tube containing 500ml of 5% activated charcoal suspension. After 30 min incubation, a sample of the supernatant fraction was taken out for scintillation counting. The protein content of the microsomes, on the other hand, was determined by using a BCA kit (Sigma Chemicals).

## 2.4 Measurement of Cell viability

Cell number was assessed by 3-(4，5-dimethylthiazol-2-yl)-2,5-diphenyl tetra-zolium bromide (MTT) staining as described by Mosmann. Briefly, MCF-7aro cells were seeded in 96-well plates and maintained in MEM supplemented with 10% charcoal dextran-treated serum (Hyclone, Logan, UT, USA). The cells were allowed 24 hr for attachment and they were treated with testosterone and/or testing compound for 48 hr. At the end of the treatment, 50μl of 1 mg/ml MTT was added to the cells and incubated at 37 ℃ for 4 hr. Cell viability was assessed with respect to the absorbance at 544 nm.

## 2.5 Transient Expression of ERK-1 in HepG2

HepG2 cells were cultured and transfected with ERK-1 expression plasmid or the empty vector similar to the reporter assay described above. After one day after transfection, total mRNA was isolated and quantified for CYP19 expression.

## 2.6 Luciferase gene reporter assay

The procedures were described previously (Po *et al.* 2002). In brief, HepG2 cells were plated in 24-well dishes. After 24 hrs, the cells were transiently transfected with 0.25μg ER-expression plasmid, 0.25μg ERE-driven reporter plasmid and 2.0ng of renilla luciferase control vector pRL (Promega, Madison, WI, USA) in LipofectAmine reagent (Invitrogen Life Technologies). After 1 day, the medium was removed and the cells were treated with different phytochemicals for 24 hrs. The cells were lysed and the activities of the luciferases were determined using Dual-Luciferase Assay Kit (Promega). The luciferase bioluminescence was quantified by using a FLUOstar Galaxy plate reader (BMG Labtechnologies GmBH, Offenburg, Germany). The ERE-driven transactivation activities represented by firefly luciferase light units were then normalized with that of renilla luciferase.

## 2.7 Measurement of gene expression by Quantitative Real Time RT-PCR Assay

Frozen tumors were pulverized in a Dounce homogenizer. Liquid nitrogen was constantly added in keeping the tissue in the frozen state throughout the homogenization. Total RNA was extracted from the sample using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The concentration and purity of RNA were determined by absorbance at 260/280 nm. 3μg of total RNA, oligo-dT, and M-MLV Reverse Transcriptase (USB Corporation, Cleveland, Ohio, USA) were used for first strand synthesis. Target fragments were quantified by real-time PCR and an Opticon™ 2 system (MJ Research, Waltham, MA, USA). Real-time PCR Master Mix Reagent kit was obtained from Applied Biosystems and PCR reactions were set up as

described in the manual. A typical reaction contained 200 nmol/l of forward and reverse primer, 2 µl cDNA and the final reaction volume was 20µl. The reaction was initiated by preheating at 50ºC for 2 min, followed by 95ºC for 10 min. Subsequently, 45 amplification cycles were then carried out with 15 s denaturation at 95ºC and 1 min annealing and extension at 58ºC. Target fragments were quantified by real-time PCR, and a DNA Engine Opticon II (MJ Research, Inc., Waltham, MA) was employed for this assay. The primers and FAM-labelled probes of CYP19 and four CYP19 exons were all purchased from Applied Biosystems (**Table 2.1**). Taqman/VIC minor groove binder probes and primers for pS2 (cat.no.Hs00170216_m1), ERα(cat.no.Hs00174860_m1)CYP19(cat.no.Hs00240671_m1),CYP17(cat.no.Hs0016 4375_m1),CYP1A1(cat.no.Hs00153120_m1),CYP1B1(cat.no.Hs00164383_m1),CO MT(cat.no.Hs00241349_m1),UGT1A1(cat.no.Hs00166592_m1),GAPDH(cat.no.Hs9 9999905_m1) (Assay on-Demand™) and real-time PCR Taqman Universal PCR Master Mix were all obtained from Applied Biosystems. PCR reactions were set up as described in the protocol, which was validated by the manufacturer. Signals obtained for GAPDH were used as a reference housekeeping gene to normalize the amount of total RNA amplified in each reaction. Relative gene expression data were analyzed using the$2^{-\Delta\Delta CT}$method (Livak and Schmittgen, 2001).

The gene expression of samples relative to control is determined by 2-ΔΔCT method (Livak & Schmittgen, 2001). The mean fold change in expression of target gene compared with control was calculated with the equation:

Relative target gene expression = $2^{-\Delta\Delta CT}$, where ΔΔCT= (CTtarget – CT house

keeping) sample - (CTtarget – CT house keeping) control.

**Table 2.1** The forward primer, reverse primer and reporter sequences of CYP19 Taqman probes (Applied Biosystems).

| | | |
|---|---|---|
| CYP19 | Assay No | CYP19-F165 |
| | Forward primer | GGAGAATTCATGCGAGTCTGGAT |
| | Reverse primer | GGAACATACTTGAGGACTTGCTGAT |
| | Reporter | TCTGGAGAGGAAACACTC |
| Exon I.1 | Assay No | EXONIA.1-JUN |
| | Forward primer | CTGTGCTCGGGATCTTCCA |
| | Reverse primer | CATCTTGTGTTCCTTGACCTCAGA |
| | Reporter | ACGTCGCGACTCTAAAT |
| Exon I.3 | Assay No | EXONI3-J68 |
| | Forward primer | AAATTAGTCTTGCCTAAATGTCTGATCACA |
| | Reverse primer | CCAAAACCATCTTGTGTTCCTTGAC |
| | Reporter | TTATAAAACAGACTCTAAATTGCC |
| Exon I.4 | Assay No | EXONI4-J60 |
| | Forward primer | GTCCCTGGCACTGGTCAG |
| | Reverse primer | CATCTTGTGTTCCTTGACCTCAGA |
| | Reporter | CCCATCAAACCAGGACTC |
| Exon II | Assay No | EXONII-J79 |
| | Forward primer | GCAACAGGAGCTATAGATGAACCTT |
| | Reverse primer | CATCTTGTGTTCCTTGACCTCAGA |
| | Reporter | CCACAGGACTCTAAATTG |
| Exon 2a | Assay No | EXONIA-J212 |
| | Forward primer | CCGCACACACAAAGCAACATTT |
| | Reverse primer | CATCTTGTGTTCCTTGACCTCAGA |
| | Reporter | CCTGTGGACTCTAAATTG |

## 2.8 Western Blotting

The pulverized samples were sonicated in lysis buffer (PBS, 1%NP40, 0.5% sodium deoxycholate, 0.1% SDS, 40 mg/L PMSF, 0.5 mg/L aprotinin, 0.5 mg/L leupeptin, 1.1 mmol/L EDTA and 0.7 mg/L pepstatin) with a cell disruptor (Branson Ultrasonics Corp., Danbury, CT, U.S.A.) on ice for 30 s. The protein concentration of tissue homogenate was determined by Dc protein assay (BioRad, Richmond, CA, U.S.A.). 30μg of homogenized protein was separated on 10% SDS-PAGE and transferred onto an Immobilon PVDF membrane (Millipore, Bedford, MA, U.S.A.). Anti-CYP19 (Abcam, Cambridge, U.K.), anti-Bcl-xL, anti-Bax, anti-Bak, anti-$p21^{CIP/WAF1}$, anti-$p57^{kip2}$, anti-cyclinA, anti-cyclinD, anti-cyclinE (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), anti-actin primary (Sigma Chem) and secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology) were used for protein detection. An ECL Detection Kit (Amersham, Arlington Heights, IL, U.S.A.) provided the chemiluminescence substrate for HRP, and the targeted protein was visualized by autoradiography. The optical density (OD) of the protein was quantified by Image J (a public domain software from NIH Image). The OD of target gene was normalized by that of actin.

MCF-7 or HepG2 cells were seeded in 6-well plate at a density of $5 \times 10^5$ cells per well. Before the day of hesperetin treatment, the cells were replaced with fresh phenol red-free RPMI-1640 medium supplemented with 5% charcoal-dextran treated FBS. The cells were treated with testing compound for 24 hrs and then harvested for protein detection. During harvesting, cells were washed twice with PBS and lysed by

0.1 ml RIPA lysis buffer (25mM Tris-HCl pH 8.8, 50mM NaCl, 0.5% NP40, 0.5% Deoxycholate, 0.1% SDS). After 10-minute incubation on ice, cells were scraped and cell lysate was collected in a 1.5-ml microfuge tube. The lysate was then subjected to sonication on ice for 30 seconds with cell disruptor (Branson Ultrasonics Corp., Danbury, CT, USA). Clear supernatant was collected by centrifugation at 13,000 rpm for 5 minutes and was transferred to a new 1.5-ml microfuge tube. Proteins were then used immediately or stored at -80˚C for long-term storage.

Protein concentration was determined by Bicinchoninic Acid (BCA) Assay (Pierce Biotechnology, Inc., Rockford, IL, USA.) with bovine serum albumin (BSA) as standards. 50μg proteins were separated by sodium dodecylsulpahte-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% resolving gel and were transblotted to Immobilon-P polyvinylidene diflupride (PVDF) membrane (Millipore Corp., Bedford, MA, USA) at 15 V for 90 minutes. The membranes were probed with primary antibodie at 4˚C overnight. The membranes were briefly washed and were then probed with appropriate secondary antibodies conjugated with horseradish peroxidase (Zymed Laboratories, South San Francisco, CA, USA) at room temperature for two hours. After probing the membranes were washed thoroughly. The antigen-antibody complexes were detected using an enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences, Piscataway, NJ, USA) and were visualized by autoradiography.

β-actin was used for protein normalization. The membrane was reprobed with anti-actin primary antibody at 1:5000 dilutions.

## 2.9 Measurement of Promoter Activity

### 2.9.1 Plasmid Preparation

The pGL-3 basic reporter vector was purchased from Promega. ERE-luciferase reporter plasmid $C_3$-LUC was a gift from Dr. Donald McDonnell (Duke University, NC, USA). ERK-1 expression plasmid was constructed by subcloning the full length cDNA of ERK-1 into the mammalian expression vector pcDNA3.1 (Invitrogen Life Technology). Several fragments localized in the 5' flanking region of exon II was investigated. These regions contain both promoter I.3 and promoter II (Zhou *et al.* 1996). The amplified genomic fragments (-556/+118 bp, -506/+118 bp -406/+118 bp, -356/+118 bp, -306/+118bp, -256/+118bp, -206/+118bp, -156/+118bp, -106/+118bp, -56/+118bp) were inserted into the pGL-3 basic reporter vector between Kpn I and Xho I restriction sites. All constructs were confirmed using restriction analysis and DNA sequencing. Primers for constructing promoter I.3/II reporter plasmid are listed in **Table 2.2.**

**Table 2.2** Primer sequences for promoter I.3/II

| Oligonucleotide | Sequences |
|---|---|
| -556 forward | ATTTGGGGTACCCTGGCCCATGGTACAAGAGA |
| -506 forward | ATTTGGGGTACCGGCCTGAGTGAGTCACTTTGA |
| -456 forward | ATTTTGGGTACCGAGAAGACCTCAACGATGCCC |
| -406 forward | GGGGGGGGTACCCTATTTTGATCAAAAAAGTCA |
| -356 forward | GGGGGGGGTACCGCCTTTTTGTTTTGAAATTGA |
| -306 forward | TTGGGGGTACCACAAAACCTGCTGATGAAGTC |
| -256 forward | GGGGGGGGTACCTTATTTTCTTATAATTTGGCA |
| -206 forward | AAAAAGGGTACCGTCACTCTACCCACTCAAGGG |
| -156 forward | TTGGGGGTACCGTCTAAAGGAACCTGAGACTC |
| -106 forward | ATGGGGGGTACCCCAAAAGATCTTTCTTGGGCT |
| -56 forward | ATGGGGGGTACCGTCTTGCCTAAATGTCTGATC |
| +118 reverse | AAACCGCTCGAGTCTTGTGTTCCTTGACCTCAG |

## 2.9.2 Transient Transfection and Dual-Luciferase Assay

$1\times10^5$ per well MCF-7 cells (or HepG2 cells) were seeded in 24-well plates for 24 h. Then transient transfection was performed using 1μl Lipofectamine and 2μl Plus reagent (Invitrogen) per well. 0.25μg reporter plasmid (containing DNA fragments derived from aromatase promoter region) with or without 0.1μg expression vector (pcDNA3.1-ERα or pcDNA3.1) were transfected into cells in

serum-free medium. The renilla luciferase vector pRL-CMV (Promega) was cotransfected as an internal correction for transfection efficiency. After 5 h incubation, cells were cultured in phenol red free PRMI 1640 supplemented with 5% charcoal-dextran treated FBS. One day after transfection, various concentrations of testing compounds with or without 100 nM E2 were added and incubated at 37°C for 24 h. The cells were lysed in lysis buffer (Promega) and stored at -80°C until assay.

Cell lysate was scraped and transferred to 1.5 ml eppendorf tube, and was centrifuged at 4°C at full speed for 5 min. 20µl of the supernatant was assayed in a dual luciferase reporter assay system (Promega). The luciferase activity was read by a FLUOstar Galaxy plate reader (BMG Labtechnologies, Offenburg, Germany), and expressed as relative light units of firefly/renilla.

## 2.10 Electrophoretic Mobility Shift Assay (EMSA)

### 2.10.1 Nuclear protein extraction

MCF-7 or HepG2 cells were seeded in 75 $cm^2$ flask and were replaced with phenol red-free RPMI-1640 medium supplemented with 5% charcoal-dextran treated FBS the day of experiment. Cells were treated with testing compound for 24 hours and nuclear protein was extracted using Novagen's NucBuster$^{TM}$ Protein Extraction Kit (EMD Biosciences, Inc., San Diego, CA, USA). Cells were washed with PBS, trypsinzed and pelleted by centrifugation at 1,000 rpm for 5 minutes. The cell pellet was resuspended in 150µl NucBuster Reagent 1 with vigorous vortexing for 15 seconds. The homogenous suspension was then chilled on ice for 5 minutes, vortexed at high speed for another 15 seconds and chilled on ice again. The cell suspension

was centrifuged at 13,000 rpm for 5 minutes at 4˚C. Supernatant was discarded and the pellet was washed with 500µl cold PBS. The pellet was then resuspended by vortexing for 15 seconds in 75µl NucBuster Reagent 2 supplemented with 1µl 100X protease inhibitor and 1µl 100 mM DTT. The suspension was then chilled on ice for 5 minutes, vortexed for another 15 seconds and chilled on ice again. Nuclear protein was collected in the supernatant by centrifugation at 13,000 rpm at 4˚C for 5 minutes and was kept at -80˚C for future experiments.

### 2.10.2 Electrophorectic Mobility Shift Assay

Complementary strands of oligonucleotides with AP-1 and CRE binding sites were commercially synthesized, annealed and labeled at 3'end with digoxigenin (DIG) using DIG Gel Shift Kit (Roche Applied Science, Indianapolis, IL, USA). Labeled probes were diluted to 1.55 fmol/µl for binding reactions. 10µg nuclear extract was mixed with 1 l DIG-labeled probe and the binding reaction was carried out at 25˚C for 30 minutes according to the following composition:

| | |
|---|---|
| 5X binding buffer | 4 µl |
| poly dI-dC (1µg/µl) | 1 µl |
| poly L-lysine (0.1µg/µl) | 1 µl |
| DIG-labeled probe (1.55 fmol/µl) | 2 µl |
| 10 µg nuclear protein | X µl |
| sterile dd$H_2O$ | 12-X µl |
| Total | 20 µl |

For supershift experiment, CRE-binding protein 1/activating transcription factor (CREB-1), C/EBP, and c-Jun(D) antibodies from Santa Cruz Biotechnology were used. The CREB-1 antibody is reactive against CREB-1, CREB, CREM-1 and ATF-1 proteins, while the c-Jun(D) antibody is reactive against c-Jun, Jun B and Jun D proteins. The antobodies were added to and incubated with the DNA-protein complex for 45 minutes at room temperature. After all incubation, the DNA-protein complexes were resolved by 5% non-denaturing polyacrylamide gel electrophoresis at 8 V/cm. The gel was then transblotted to a positively charged nylon membrane at 15 V for 60 minutes and the membrane was then UV cross-linked 3 times at 120 mJ. The membrane was soaked in Blocking Buffer at room temperature for 1 hour, probed with anti-DIG antibody for 30 minutes at room temperature and then washed in Washing Buffer 3 times. The membrane was then equilibrated in Detection Buffer for 10 minutes at room temperature and the DNA-protein complexes were detected by CSPD® solution. Chemluminescence was enhanced by incubation at 37˚C for 10 minutes and was detected by autoradiography.

## 2.11 Serum E2 determination

Blood samples were collected by cardiac puncture at the time of sacrifice and placed into heparin-containing tubes and centrifuged at 500 g for 5 min. Plasma samples were stored at -80°C until analyzed. The serum estradiol was measured using Estradiol ELISA kit according to the company's protocol (Cayman Chemical Company, Ann Arbor, Michigan USA). Serum samples (50µl) were added into a 96-well plate coated with antibody raised against estradiol. After 1-hour incubation at

room temperature, the plate was washed and then developed in the dark for 60-90 minutes. Finally the plate was read at 410 nm using a plate reader (BMG Labtechnologies GmBH, Offenburg, Germany). The amount of estradiol in serum was quantitated by competing with estradiol tracer (estradiol linked to an acetylcholinesterase) for binding to the antibody. The absorbance representing the amount of serum estradiol could be read against a standard curve constructed with the estradiol provided in the kit.

## 2.12 Statistical Analysis

For the *in vitro* studies, the data will be analyzed by the software package Prizm5 (GraphPad Software, Inc., San Diego, CA). One Way ANOVA with Tukey's posthoc test was used for multi-group comparisons. Another software package SigmaPlot (SPSS Inc., Chicago, IL, USA) was used for graphing the Lineweaver-Burk plots. For the mouse study, Two Way ANOVA was used for analyzing data obtained on tumor volume growth.

# CHAPTER 3
# EFFECT OF DIOXIN EXPOSURE ON AROMATASE EXPRESSION IN OVARIECTOMIZED RATS

## 3.1 INTRODUCTION

Dioxins are a group of polyhalogenated aromatic hydrocarbons that are stable and bioaccumulative. They are aryl hydrocarbon receptor (AhR) agonists and display weak estrogenic properties (Ohtake *et al.* 2003). Exposure of dioxins may lead to developmental and reproductive abnormalities, and cancer (Birnbaum and Tuomisto 2000). However, its mechanism of toxicity has not been clearly established (Weiss *et al.* 2007).

Human aromatase is a 55-kDa protein encoded by a single-copy gene with 10 exons (Means *et al.* 1989; Toda *et al.* 1990). The promoter utilization for cyp19 regulation varies in different tissues, which provides the basis for tissue-specific expression (Harada *et al.* 2003). The coding region extends from Exon II to IX, and a number of untranslated segments in Exon I dictate the gene regulation. Messenger RNAs are transcribed from one segment in Exon I splicing onto a common acceptor site in Exon II. Since each of the segments in Exon I is associated with a unique promoter region, tissue-specific regulation of aromatase expression is achieved through the use of these alternate promoters (Simpson *et al.* 1997). Nevertheless, there is only one common protein product translated because the protein coding regions of the transcripts are identical regardless of the tissue site of expression. In

rodents, cyp19 is expressed in the gonads and the brain. There are only two alternate splicing species identified: the gonadal-specific first exon II and the brain specific exon If (Honda *et al.* 1996; Yamada-Mouri *et al.* 1996).

The association between 2,3,7,8-tetrachlorodibenzo-para-dioxin (TCDD) exposure and breast cancer risk has been inconclusive in epidemiological studies (Brown *et al.* 1998; Revich *et al.* 2001; Warner *et al.* 2002), and animal models have also revealed contradictory findings. Some studies (Brown *et al.* 1998; Desaulniers *et al.* 2001) have demonstrated that TCDD treatment increases the number of 7,12-dimethylbenz[a]anthracene (DMBA) or methylnitrosourea (MNU)-induced mammary tumor in rats. On the contrary, others claim TCDD to be antiestrogenic and chemopreventive against spontaneous breast cancer (reviewed in Greenlee *et al.*, 2001; Safe, 2001).The differences could be attributed to the timing of exposure, and the estrogen-dependence of the cancerous tissue.

TCDD can decrease aromatase protein or mRNA in JEG-3 cells (Drenth *et al.* 1998) and granulose cells (Dasmahapatra *et al.* 2000; Moran *et al.* 2000). The inhibition could be the result of reduced substrate supply rather than direct enzyme interaction (Moran *et al.* 2000). In contrast, others have observed that the toxicant induces aromatase in human placental cells (Augustowska *et al.* 2003) and primary Sertoli cells (Lai *et al.* 2005).

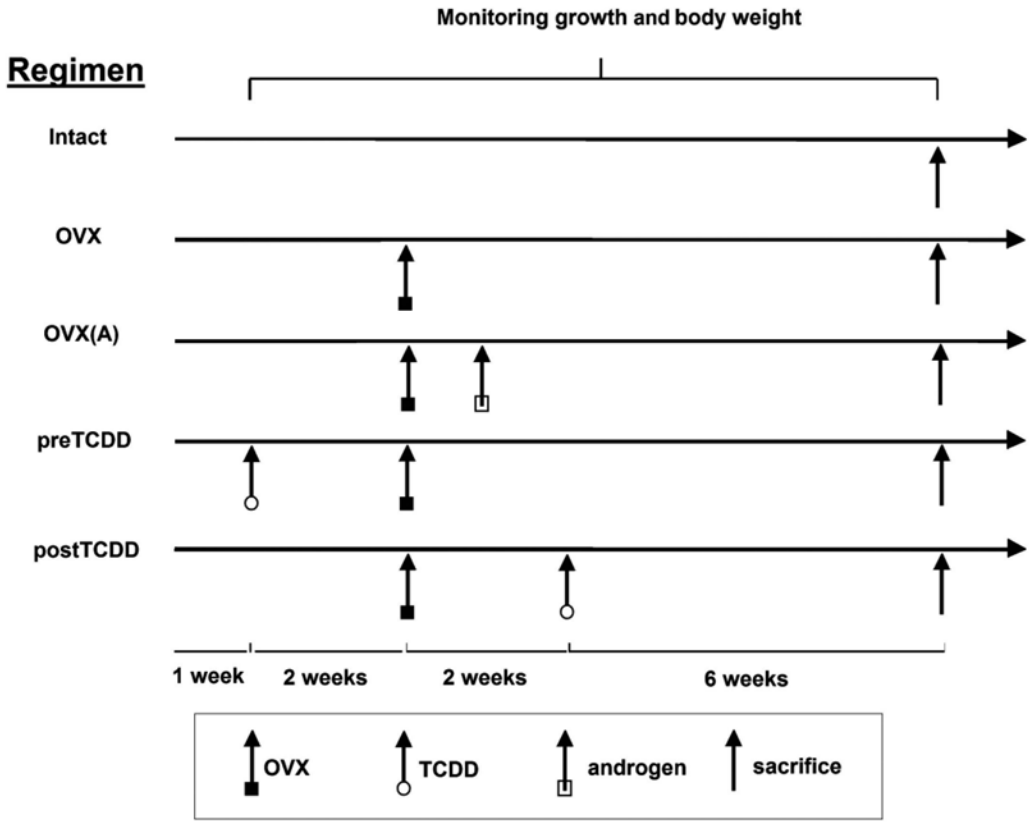
The estrogenic and antiestrogenic effect of TCDD could be determined by the absence or presence of estrogen in the system. As illustrated by (Boverhof *et al.* 2006), dioxins induce estrogen receptor (ER)-dependent gene expressions in murine uterus

upon ovariectomy. The induced expressions could be driven by increased systemic estrogen concentration rather than the direct action of the toxicant. Rat models have been widely used to evaluate the toxicity of environmental carcinogens or contaminants (Desaulniers *et al.* 2001) . In order to examine whether estrogen metabolism was altered at ovariectomy under TCDD exposure, we employed a rat model to address this issue in the current study and the animal design was shown as below. As aromatase is mainly expressed in the brain, adipose tissues and gonads, we mainly studied the expression in these tissues (Sebastian and Bulun 2001; Zhao *et al.* 2005). As induced ER-dependent gene expressions have been demonstrated in Boverhof's study, we hypothesized that expression of the rate-determining enzyme cyp19 could be increased by TCDD.

**Animal experimentation** Female Sprague-Dawley (SD) rats (about 3-week old) were acquired from and housed (2 per cage) in a 12/12 h light-dark daily cycle at Laboratory Animal Services Centre, Chinese University of Hong Kong. They were fed Prolab® 5P76 Isopro® 3000 diet and tap water throughout the experimental period. The protocol adopted in the present study was approved by Animal Experimentation Ethics Committee of Chinese University of Hong Kong, reference no. 07/038/ERG and 470007.

Fifty rats were divided into five groups under the regimen as shown in the diagram below (n = 10 per group). Range of single dose from 0.1 to 10μg/kg was used in many studies before. The dosage selected in this study was 1/10 of the $LD_{50}$ concentration of TCDD (Salisbury & Marcinkiewicz, 2002), and its timing effect

was examined. Ovariectomy (OVX) or sham operation (Intact) was performed at 42 days of age. TCDD (2.5 μg/kg body weight in corn oil) in a single dose was administered before (pre-TCDD) or after ovariectomy (post-TCDD), while all other rats were gavaged with corn oil. Previous study has shown that reduced cyp19 expression may be induced by inadequacy of cyp19 substrate (Abdelgadir et al., 1994). Pellets (25 mg androstenedione mixed with 12.5 mg cholesterol) were implanted *s.c.* in rats under the regimen OVX(A) following ovariectomy. Their body weight was monitored every week. Rats were sacrificed by cervical dislocation, and blood was collected by cardiac puncturing. Brain and abdominal adipose tissues were frozen in liquid nitrogen and stored at –80°C. Previous study (Simpson et al., 1994) has documented the expression of aromatase in the abdominal adipose tissue. In addition, uterus and liver weights were measured at the day of sacrifice.



## 3.2 RESULTS

### 3.2.1 Effect of TCDD and ovariectomy on rat body weight

The rat body weights were measured weekly since checked out from the animal facility. No significant differences in body weight were observed among the ovariectomized rats treated with TCDD or androstenedione. Disregard the treatments, all the ovariectomized rats appeared to be significantly heavier than Intact rats 4 weeks after the operation (**Fig.3.1**). By the time of sacrifice, the body weight of OVX rats were about 1.3-fold of Intact rats.

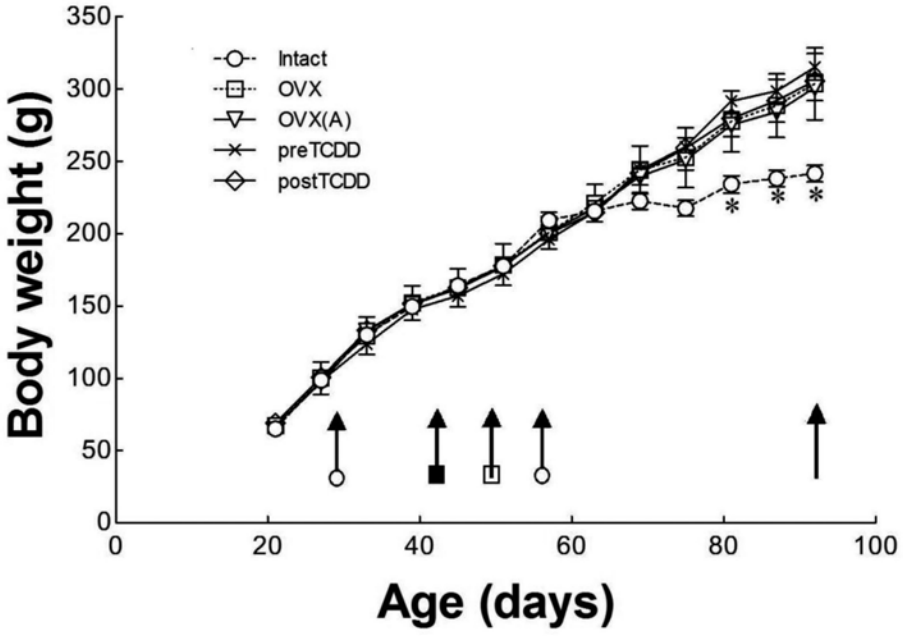
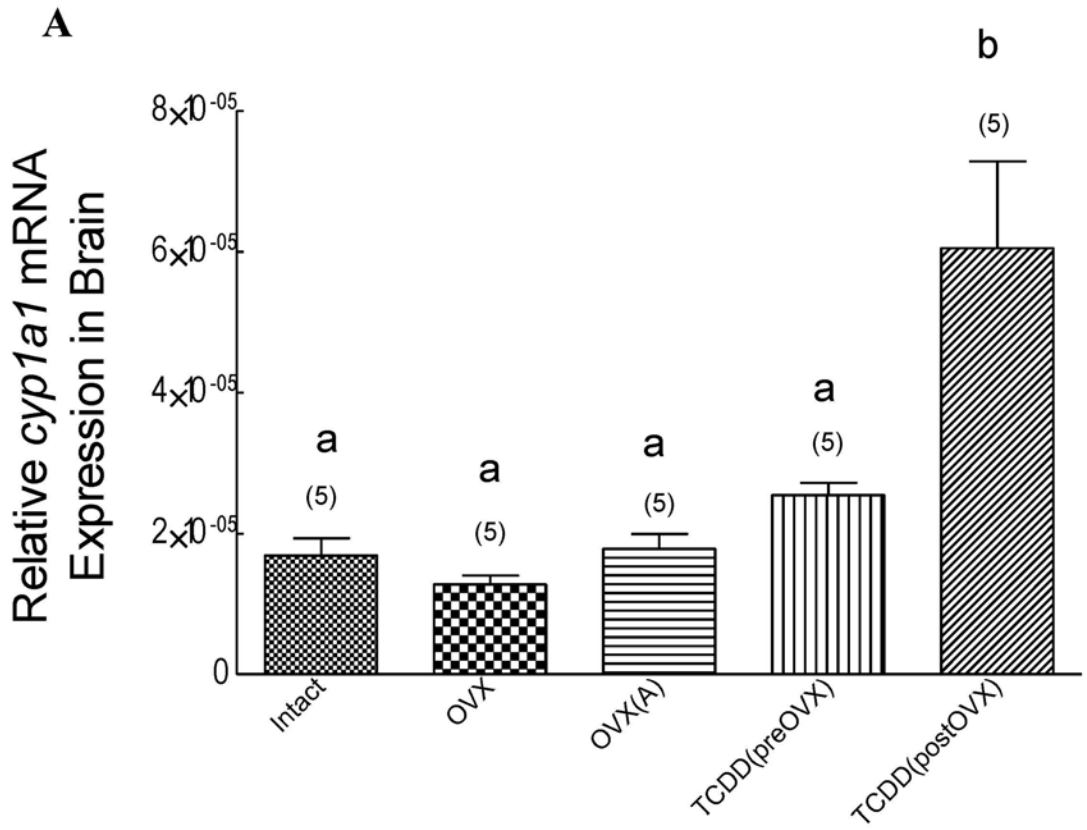



**Fig.3.1.** Effect of TCDD and ovariectomy on body weight. Female SD rats were treated with TCDD before and after ovariectomy. Their body weights were monitored. Values are means ± SEM. The data collected in the same day was analyzed by One-way ANOVA, followed by Tukey's Multiple Comparison test. Means labeled with (*) are significantly different from the other groups.

### 3.2.2 TCDD induced Cyp1a1 mRNA expression in rat brain and adipose tissue

The expression of cyp1a1 was induced by almost 6-fold and 100-fold in the brain (**Fig. 3.2A**) and adipose (**Fig.3.2B**) tissues respectively in the post-TCDD group. The expression measured in rats received TCDD before ovariectomy (pre-TCDD), on the other hand, appeared to be marginally higher than the others without TCDD treatment in the two tissues. The result suggested that induction of cyp1a1 expression could subside over time.


A
Relative cyp1a1 mRNA Expression in Brain
8x10-05
6x10-05
4x10-05
2x10-05
0
a
(5)
a
(5)
a
(5)
a
(5)
b
(5)
Intact
OVX
OVX(A)
TCDD(preOVX)
TCDD(postOVX)

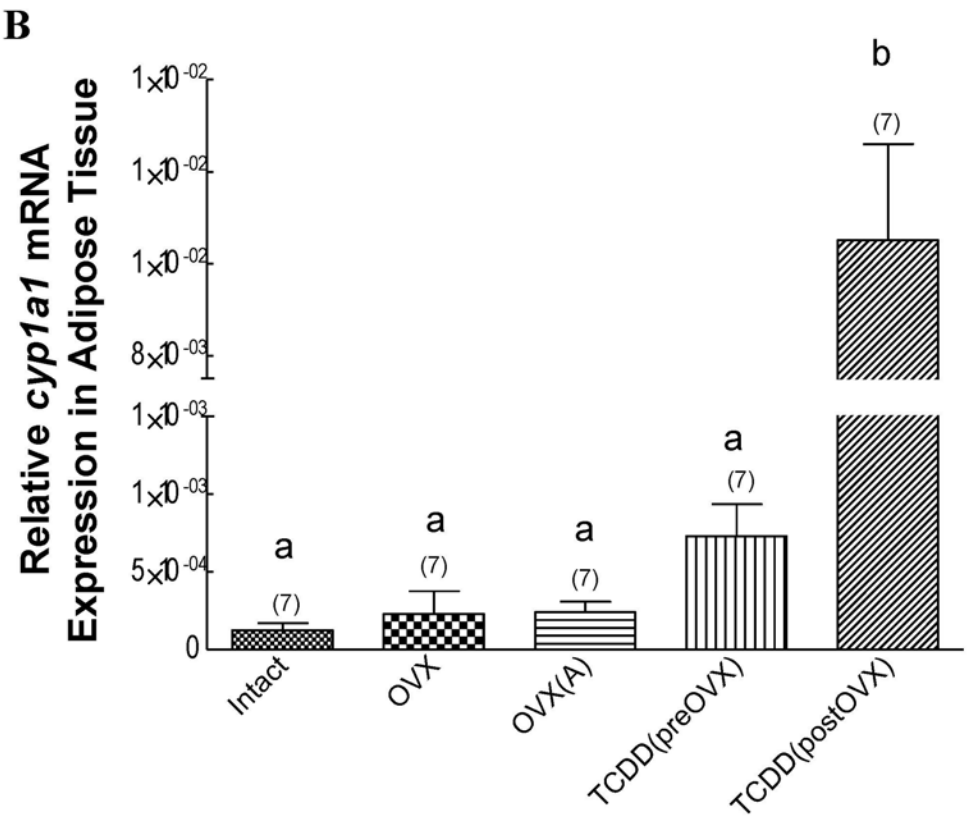



**Fig.3.2.** TCDD induced cyp1a1 mRNA expression in brain and adipose tissue. Female SD rats were gavaged with TCDD or vehicle at pre- or post-ovariectomy and total mRNA was extracted from brain (A) and adipose tissue (B). Messenger RNA expression of cyp1a1 was quantified by real-time PCR. Values are means ± SEM, the numbers of samples are given in the parentheses. The data was analyzed by One-way ANOVA, followed by Tukey's Multiple Comparison test. Means labeled with different letter are significantly different.

### 3.2.3 Differential effect of TCDD on cyp19 mRNA expression in brain and adipose tissue

TCDD given to rats before ovariectomy (pre-TCDD) suppressed cyp19 expression in the brain by 50% (**Fig.3.3A**). However, the reverse was observed in the adipose tissue where the relative amount of transcripts was increased by about 3-fold (**Fig.3.3B**). Assaying in the two tissues revealed that cyp19 expression in the brain was 1000 fold higher than that in the fat pad. In rats with androstenedione implant (OVX(A)), cyp19 expression was not affected in these two tissues. The result suggested that cyp19 expression was not upregulated by the substrate of the enzyme.

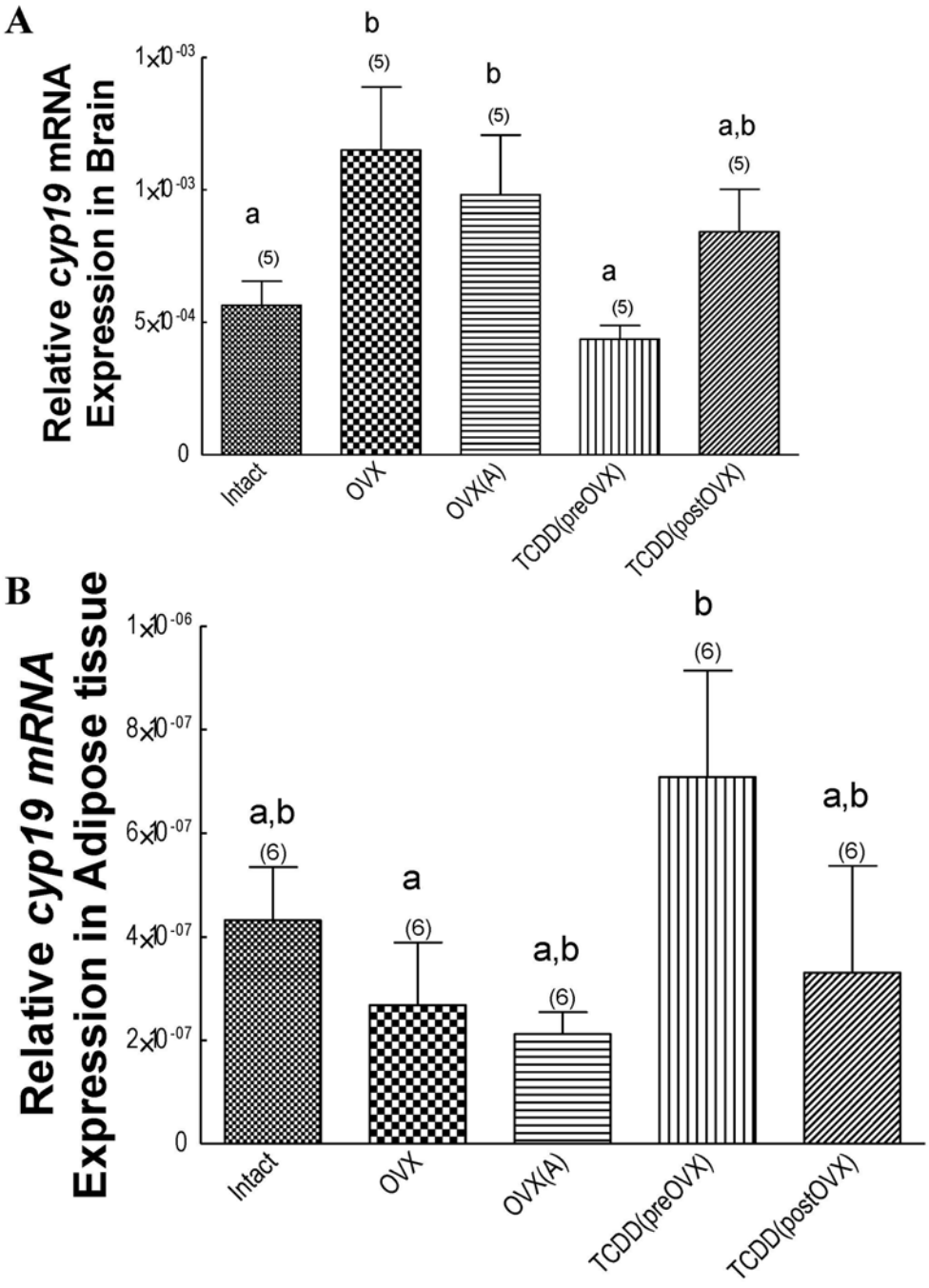
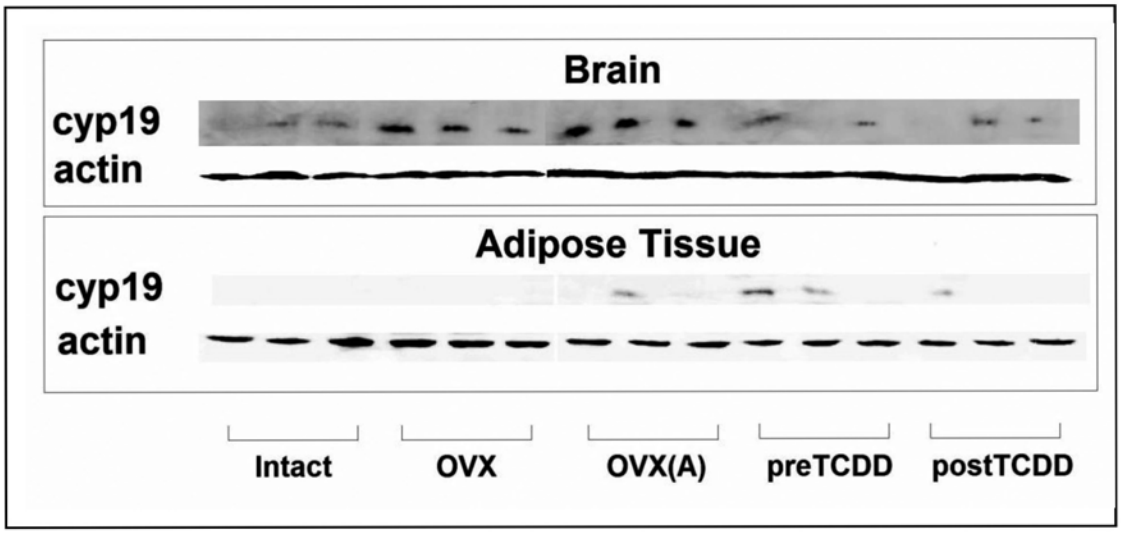



**Fig.3.3.** Effect of TCDD on cyp19 mRNA expression in brain and adipose tissue. Female SD rats were gavaged with TCDD or vehicle at pre- or post-ovariectomy. Total mRNA was extracted from brain (A) and adipose tissue (B). Messenger RNA expression of cyp19 was quantified by real-time PCR. Values are means ± SEM, the numbers of samples are given in the parentheses. The data was analyzed by One-way ANOVA, followed by Tukey's Multiple Comparison test. Means labeled with different letters are significantly different.

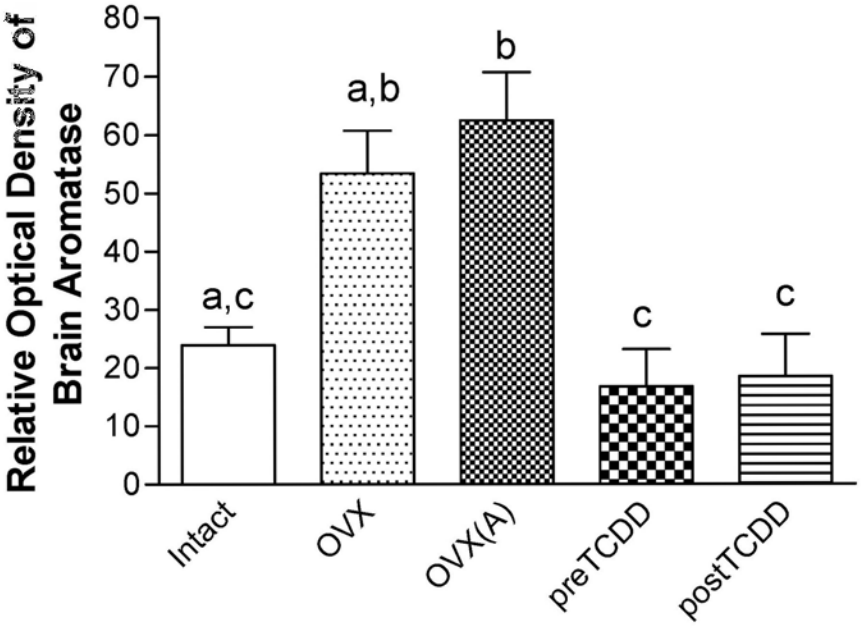
### 3.2.4 Protein expression of cyp19 in TCDD-treated rats

The differential expression of cyp19 transcripts prompted the examination on the protein amount. Western analysis in **Fig.3.4A** indicated that OVX rats had higher brain cyp19 protein than TCDD-treated rats. The cyp19 protein content in adipose tissue was the opposite of the brain, although the amounts were barely detectable in the samples. The optical density (OD) of the protein images was then measured. Cyp19 protein expression under pre-TCDD appeared to be higher in adipose tissue (**Fig.3.4B**) and lower in brain (**Fig.3.4C**) according to the quantified OD readings. OVX(A) rats maintained the OD reading at comparative levels as OVX group in both tissues.

A


Brain
cyp19
actin
Adipose Tissue
cyp19
actin
Intact
OVX
OVX(A)
preTCDD
postTCDD

B


Relative Optical Density of Brain Aromatase
80
70
60
50
40
30
20
10
0
a,c
a,b
b
c
c
Intact
OVX
OVX(A)
preTCDD
postTCDD

C

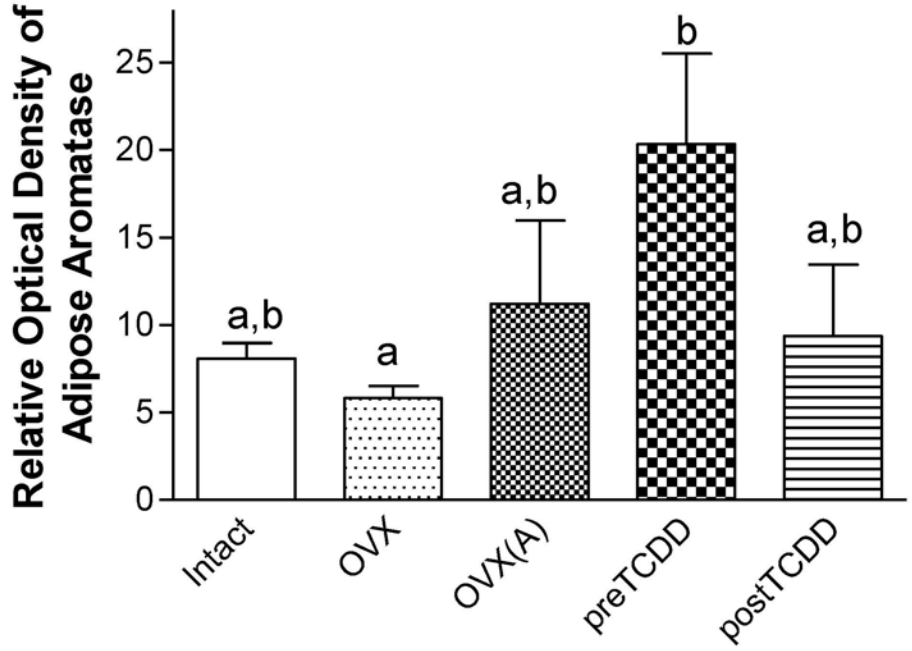
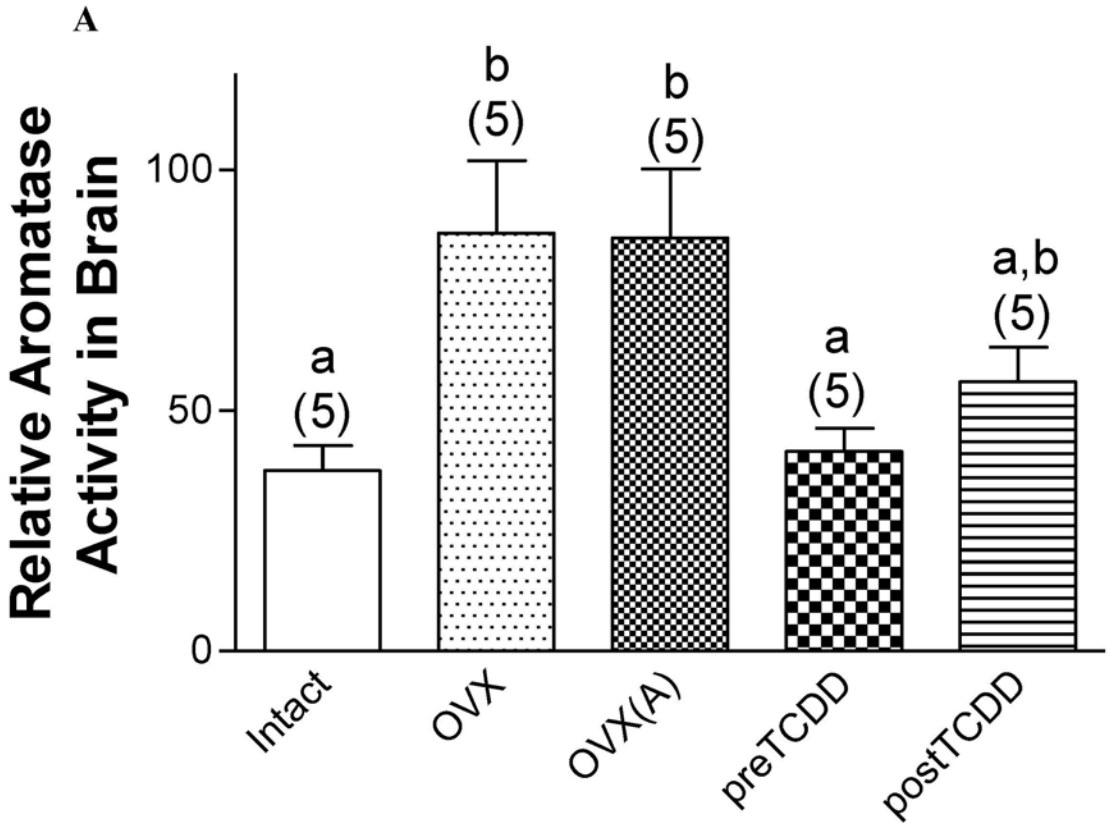



**Fig.3.4.** Western blot analysis of brain and adipose tissue cyp19 in TCDD-treated rats. Tissues were homogenized and protein samples were extracted from the tissues (A): Lanes 1–3, Intact; 4–6, OVX; 7–9, OVX(A); 10–12, pre-OVX; 13–15, post-OVX. Optical density of the cyp19 protein image in brain (B) and adipose tissue (C) was measured. The data was analyzed by One-way ANOVA, followed by Tukey's Multiple Comparison test. Means labeled with different letters are significantly different.

### 3.2.5 Aromatase activity in brain and adipose tissues

The aromatase activities in the brain of OVX and OVX(A) rats were two times higher ($p$<0.05) than those of Intact and pre-TCDD rats (**Fig.3.5A**). On the contrary, the aromatase activity in the adipose tissue of OVX rats was about half of that of the pre-TCDD rats (**Fig.3.5B**). The activities were consistent with the expression results.



**B**

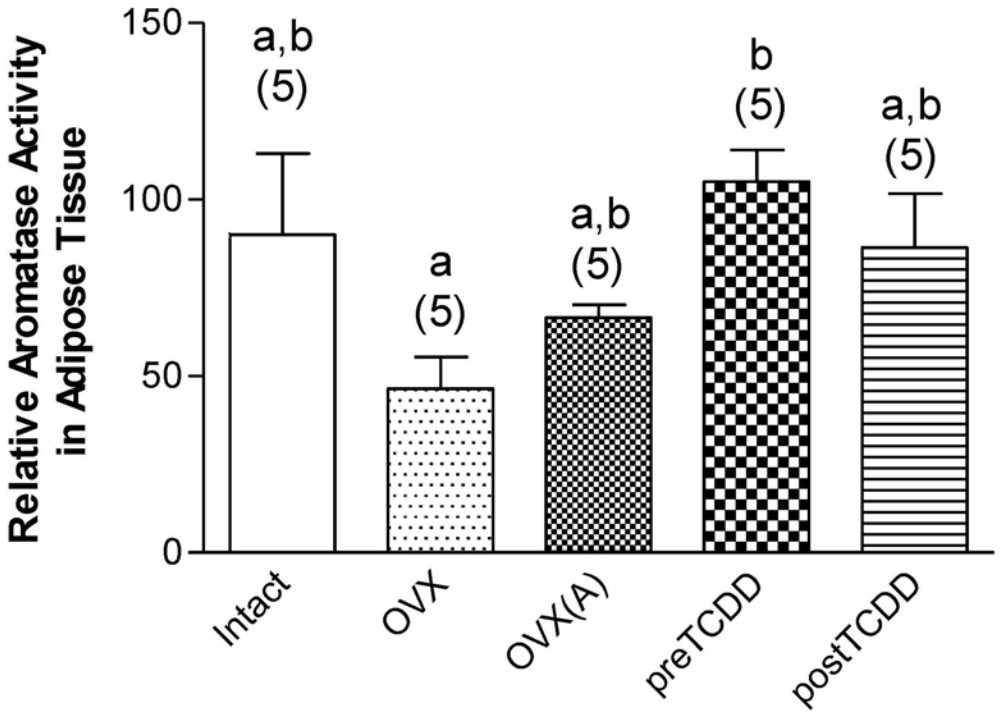



**Fig.3.5.** Microsomal aromatase activities assayed in rat brain and adipose tissues under the influence of TCDD. Aromatase activities were determined in the microsomes isolated from the brain (A) and adipose tissue (B).Values are mean s± SEM, the numbers of rat samples are given in the parentheses. The data was analyzed by One-way ANOVA, followed by Tukey's Multiple Comparison test. Means labeled with different letters are significantly different.

### 3.2.6 Plasma estrogen level in rats treated with TCDD

Serum collected from rats with pre-ovariectomy TCDD treatment (pre-TCDD) displayed a significant increase of estradiol concentration compared to OVX rats (**Fig.3.6**). This result demonstrated that TCDD administered before ovariectomy could increase serum estrogen concentration. However, rats received androstenedione OVX(A)) or TCDD after ovariectomy (post-TCDD) maintained similar levels of serum estrogen as OVX rats.

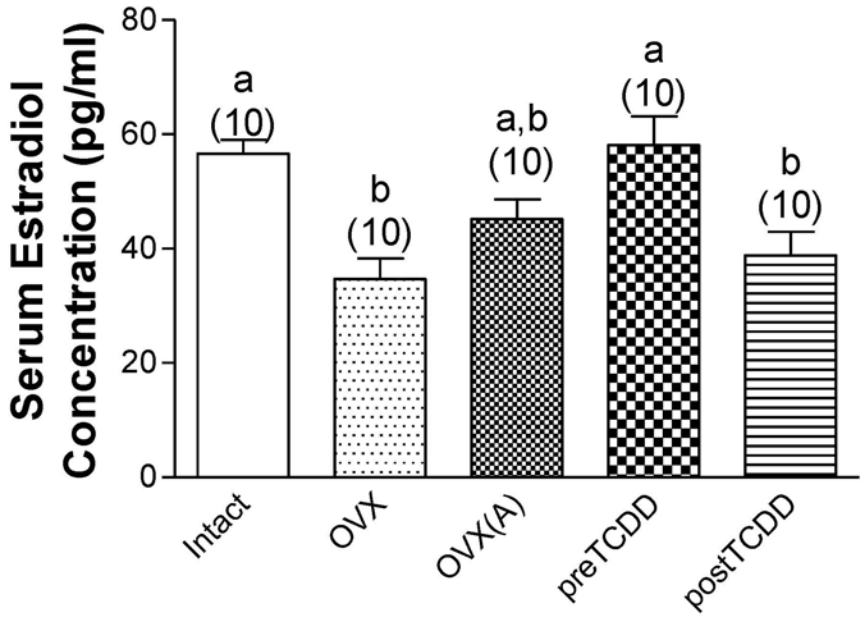



**Fig.3.6.** Serum estradiol concentration in rats treated with TCDD. Blood was drawn from the animals at euthanization. Serum estradiol concentration was quantified by ELISA. Values are means ± SEM, the numbers of rat samples are given in the parentheses. The data was analyzed by One-way ANOVA, followed by Tukey's Multiple Comparison test. Means labeled with different letters are significantly different.

### 3.2.7 Effect of TCDD on uterine weight in ovariectomized rats

In comparison to OVX, pre-TCDD rats had a higher mean uterine weight as shown in **Fig.3.7**. Uterine weight in post-TCDD was not significantly different from that of OVX. This trend of uterine weight was consistent to the corresponding serum estradiol concentration.

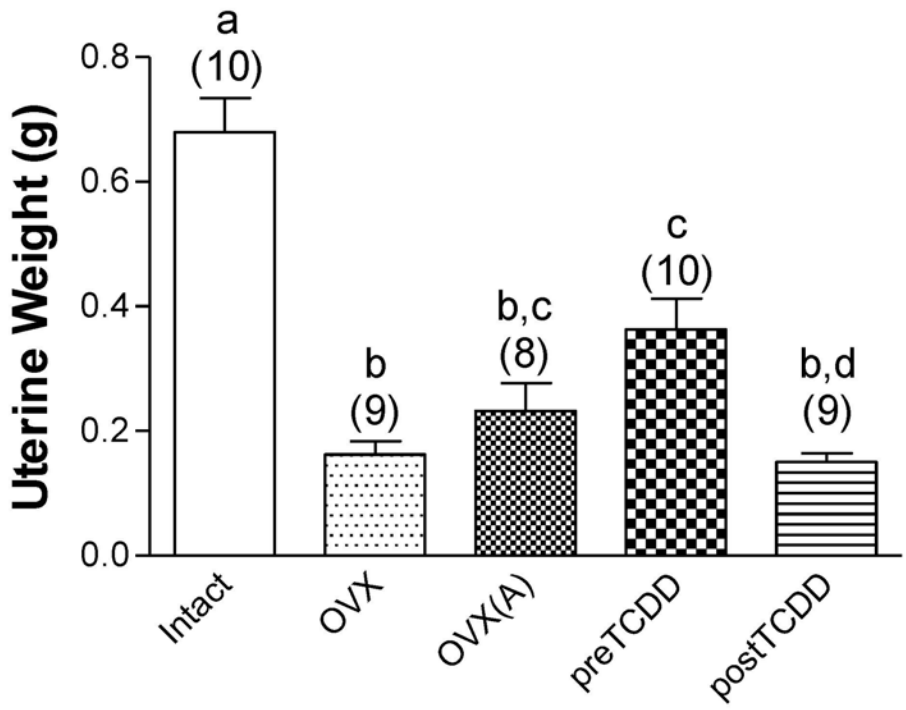



**Fig.3.7.** Uterine weight in ovariectomized rats under TCDD treatment. Uteri of the experimental animals were dissected and weighed at euthanization. Values are means ± SEM, the numbers of samples are given in the parentheses. The data was analyzed by One-way ANOVA, followed by Tukey's Multiple Comparison test. Means labeled with different letters are significantly different.

## 3.3 DISCUSSION

In the present study, we demonstrated that pre-ovariectomy administration of TCDD could reduce cyp19 expression and activity in rat brain. The toxicant also tended to raise the cyp19 expression and activity in adipose tissue. TCDD administered after ovariectomy appeared to have null effect on cyp19 expression in brain. Because a single dosage had been given to the rats, either life-cycle timing or delayed effect was suggested in the present study. On the other hand, the result of cyp1a1 expression did not reveal any connection between AhR or its downstream genes and *cyp19* transcription.

Paquette *et al* (2007) have shown that ovariectomy would increase the body weight of rats through subcutaneous fat accumulation. They further illustrate that estrogen replenishment can fully erase the body weight increase in ovariectomized rats. These observations are consistent with our results between OVX and Intact rats. Ovariectomy reduces uterine weight, and administration of exogenous estrogen may restore the uterine weight (Lea *et al.* 1999). The uterine wet weight result in the current study appeared to be proportional to the corresponding serum estradiol concentration. Other ovarian hormones might also contribute to the uterine weight, since the mean uterine weight of Intact rats were two times greater than that of pre-TCDD rats with comparable serum estradiol concentration.

Implanting androstenedione did not increase the plasma estradiol level in rats under ovariectomy as compared to OVX rats. This might illustrate that the substrate pool in brain was not a limiting factor for the estradiol production. Or estrogen

synthesized in the brain was utilized locally. Ineffective transporter system moving the hormones across the blood-brain barrier was also a possibility.

With regard to the cyp1a1 expression, rats received TCDD administered before ovariectomy (pre-TCDD) did not show a significant increase as compared to control rats (OVX). The half-life of TCDD is around 3 weeks, so the toxicant might be mostly eliminated from the body over the time span and the TCDD-responsive gene expression was returned to the baseline. Cyp1a1 facilitates the hydroxylation of estrogen to form 2-hydroxyestrogen. In the present study, serum estradiol concentration was not reduced in post-TCDD rats when adipose cyp1a1 was highly expressed. TCDD itself would dock to cyp1a1 active site (Prasad *et al.* 2007) and prevent the enzyme from taking up estradiol. Moreover, the cross-reactivity of the antibody against estradiol might not be able to exclude the hydroxylated estrogen.

Zhao *et al.* (2005) have found that the circulating estrogen level and the aromatase expression in liver and abdominal adipose tissue are gradually increased in ovariectomized rats. Another study has shown that ovariectomy may increase cyp19 expression in rat pituitary and extrinsic estradiol administration can downregulate the induced expression (Galmiche *et al.* 2006). On the other hand, castration would downregulate the cyp19 expression in the brain and androgen supplementation can restore the expression in rats (Abdelgadir *et al.* 1994).These studies illicit the sex differences in cyp19 expression. In the current investigation an elevated cyp19 expression in rat undergone ovariectomy was consistent to the Galmiche study, while androgen administration could not reverse the increase.

When female rats are exposed to TCDD in utero and during lactation, inhibition of brain aromatase activity is demonstrated and demasculinization may result (Ikeda *et al.* 2005). The toxicant also reduces aromatase mRNA level and activity in the ovaries of infant pups (Myllymaki *et al.* 2005), although negative results are observed by (Pesonen *et al.* 2006). These *in vivo* studies have illustrated the complexity of the gene regulation of aromatase. The present study suggested that timing of exposure and tissue specificity were two critical factors in determining the effect of TCDD on the aromatase gene.

Several regulatory elements have been identified in the proximal promoter of rat cyp19. The expression can be regulated by the DNA-binding regions like cAMP response element-like sequence (CLS), nuclear receptor elements half sites (NREs), GATA binding site, Yin-yang-1 (YY1) response element, and an activation protein 3 (AP3) binding site (Stocco *et al.* 2007). TCDD can activate cAMP-response element-binding (Vogel *et al.*, 2004), which could be a potential pathway for the induction of aromatase expression in adipose tissue in the present study.

Although TCDD does not bind to estrogen receptor (Klinge *et al.* 1999), it has weak estrogenic activity and activates genes responded to estrogen in murine uterus (Watanabe *et al.* 2004; Boverhof *et al.* 2006). The induction of extragonadal aromatase as shown in current findings could be the underlying mechanism. Results of the present study further suggested that pre-ovariectomy administration of TCDD increased the circulatory estrogen by upregulating extragonadal cyp19 expression. It has been reported the brain cyp19 expression was regulated via

hypothalamic-pituitary-gonadal axis previously (Galmiche *et al.* 2006). The estrogen then feedback inhibited the brain cyp19 expression in the hypothalamic-pituitary-gonadal axis.

# CHAPTER 4
# DIETARY ADMINISTRATION OF THE LICORICE FLAVONOID ISOLIQUIRITIGENIN DETERS GROWTH OF MCF-7 CELLS OVEREXPRESSING AROMATASE

## 4.1 INTRODUCTION

Exposure to estrogen has been considered a risk factor for breast cancer (Colditz 1999; Key *et al.* 2002). This causal effect of estrogen exposure was supported in a transgenic model performed recently (Yoshidome *et al.* 2000). Estrogen can be synthesized from cholesterol in several steps involving cytochrome P450 (CYP) 19, and it can be hydroxylated into several metabolites. Among the metabolites, 4-hydroxyestrogen retains the cell proliferative properties of estrogen and can be further metabolized into carcinogenic moiety as demonstrated in animal models (Liehr *et al.* 1986).

In spite of its mutagenic potential, estrogen has long been regarded as a cancer promoter. It induces proliferation of breast cancer cells and alters Bcl-2 family protein expressions to the favor of anti-apoptosis (Leung and Wang 1999). Although alternate pathways have been suggested, the receptor-mediated nuclear event is still the core of the hormone's physiological action. Therefore, the antiestrogen tamoxifen is usually administered as an adjuvant therapy for receptor-positive breast cancers. Alternatively, CYP19 (aromatase) inhibitors can be administered for purposes of targeting estrogen reduction. One study has found that CYP19 inhibitors

can be more effective than tamoxifen in protecting against the development of contralateral breast cancers (Kudachadkar and O'Regan 2005).

CYP19 inhibition is a contemporary treatment for breast cancer. Increased CYP19 expression has been demonstrated in breast cancer tissue, and estrogen concentration in the tissue is many times higher than the circulation (Miller and Dixon 2001). Santner et al. (Santner *et al.* 1993) and Yue *et al.* (1998) have illustrated that locally produced estrogen encourages tumor growth. Lee *et al.* (2003) and Hirose *et al.* (2004) have shown that polymorphisms in the *CYP19* gene are associated with increased risk of breast cancer.

The promoter utilization of human aromatase gene is tissue-specific and promoters I.3 and II have been identified as responsible for its expression in breast cancer cells (Chen *et al.* 1999). Many factors have been described for the regulation of the transcription of aromatase. Cyclic AMP, phorbol esters, dexamethasone, prostaglandin (PG) E2, transforming growth factor-β, and γ-interferon are compounds found to increase transcriptional activity (Simpson *et al.* 1997). In contrast, cyclooxygenase inhibitors exhibit inhibitory effect on *CYP19* expression and enzyme activity (Diaz-Cruz *et al.* 2005).

Licorice is the sweet-tasting rhizomes of a bean plant and quite commonly used in Western countries for culinary purposes, while it is a medicinal herb in China. Many flavonoids have been isolated from licorice, and their pharmacological properties may be applicable in preventive medicine. Isoliquiritigenin (ILN) is a flavonoid isolated from licorice. Previous research has demonstrated its

vasorelaxation induction in rat aorta (Yu and Kuo 1995), and anti-platelet aggregation *in vitro* (Tawata *et al.* 1992). ILN can also inhibit lipopolysaccharide (LPS)-induced COX-2 expression in RAW264.7 mouse macrophages (Takahashi *et al.* 2004). Studies have also illustrated ILN's protection against DMBA-induced skin carcinogenesis (Yamamoto *et al.* 1991) and azoxy-methane-induced colon carcinogenesis (Baba *et al.* 2002).

MCF-7 cells are commonly used in breast cancer research. However, they express a limited amount of aromatase as compare to most estrogen-responsive tumors (Miller and Dixon 2001), and ovariectomized nude mice transplanted with CYP19-overexpressing MCF-7 (MCF-7aro) cells has been developed as a model for studying postmenopausal breast cancer (Yue *et al.* 1994). In the present study, effect of ILN on aromatase *in vivo* and *in vitro* was investigated. The aforesaid post-menopausal breast cancer model was employed for the study on enzyme inhibition. In the second part, the molecular mechanism of ILN on the transcriptional control of CYP19 was addressed in wild-type MCF-7 cells.

## 4.2 RESULTS

### 4.2.1 Enzyme inhibition assay performed on MCF-7aro cells and recombinant protein

Previous study (Wang *et al.* 2006) has shown that MCF-7aro cells can be used for enzyme inhibition analysis. ILN displayed an inhibitory effect with an $IC_{50}$ value about 2.5 μM in the MCF-7aro cells (**Fig.4.1)**. The enzyme inhibition was further confirmed in the recombinant enzyme system (human CYP19 Supersomes®, BD Gentest, Woburn, MA) and the $IC_{50}$ value was determined to be 3.8 μM (**Fig.4.1**).

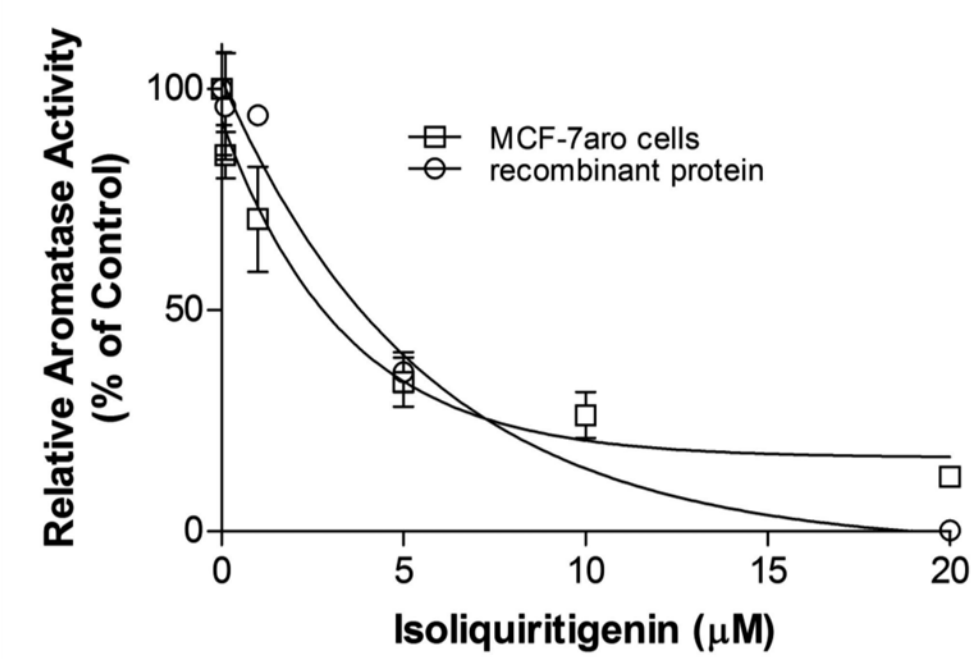
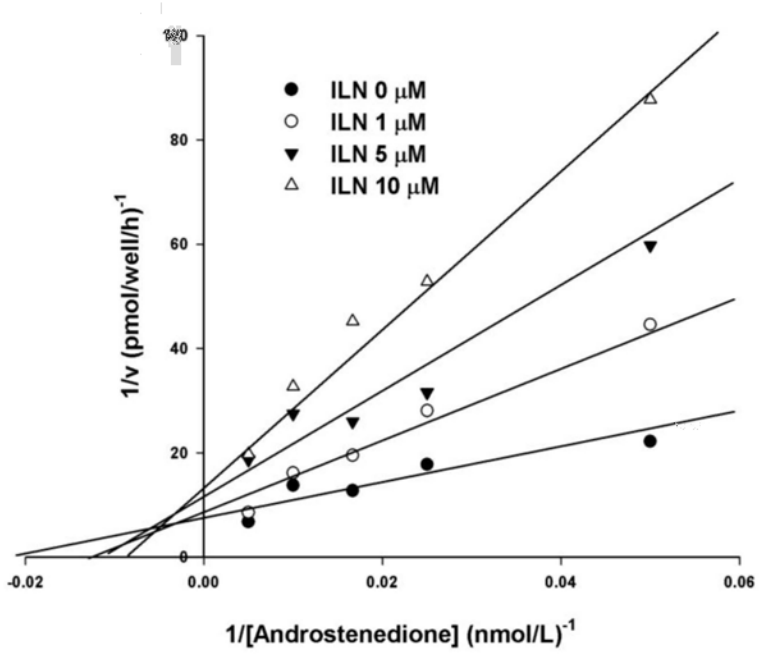



**Fig.4.1** Inhibitory effect of isoliquiritigenin on aromatase activities. In recombinant protein, isoliquiritigenin could inhibit aromatase activity with an $IC_{50}$ value of about 3.8 μM comparable to the value 2.5μM determined in MCF-7aro cells. One-way ANOVA followed by Bonferroni's Multiple Comparison Test if significant differences ($p < 0.05$) were observed. Values are means ±SEM.

### 4.2.2 Aromatase Inhibition Kinetic Assay of Isoliquiritigenin

To estimate the inhibition type of ILN on aromatase, enzyme kinetic analysis was also performed on MCF-7aro cells. Concentration of [1β-$^{3}$H(N)]-androst-4-ene-3,17-dione ranged from 20 to 200 nM were employed. Four concentrations, i.e. 0, 1, 5, and 10 μM, of ILN were selected for kinetic analysis. Lineweaver-Burk plot (**Fig. 4.2.A**) showed that ILN had a mixed type of inhibition on CYP19. *Ki* value was estimated to be 2.8 μM as shown in the subplot (**Fig.4.2.B**).

**A**




**B**

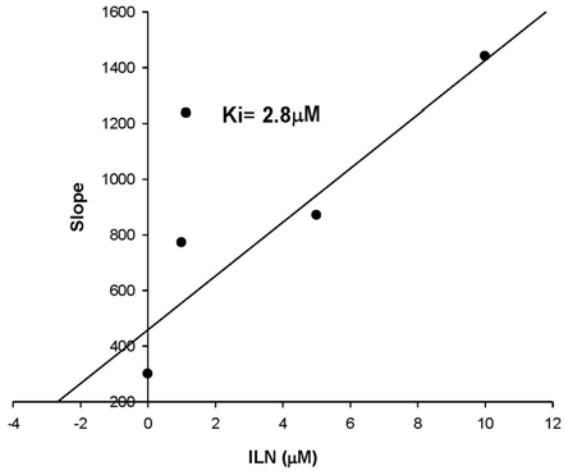



**Fig.4.2**. Aromatase inhibition kinetic analysis in MCF-7aro cells by isoliquiritigenin. MCF-7aro cells were cultured and assayed for aromatase. Four concentrations, 0, 1, 5, and 10 □M, of isoliquiritigenin were administered for the enzyme kinetic assay. Lineweaver-Burk plot results showed that isoliquiritigenin had a mixed type of inhibition on CYP19 **(A)**. The *Ki* value, which was derived from the slopes of the enzyme kinetic data, was about 2.8 μM as shown in the subplot **(B)**. Values are means ±SEM.

### 4.2.3 Specific inhibition on testosterone-induced proliferation in MCF-7aro cells

ILN was able to reduce the testosterone-induced proliferation of MCF-7aro cells through the inhibition of aromatase (**Fig.4.3**). The administration of 10nM testosterone increased the cell number by 60% as shown at 0μM of ILN. At 5μM, ILN could bring down the testosterone-induced cell growth to a level comparable to their testosterone-less counterparts. The lower panel indicated that the over-expressed aromatase protein did not vary among various treatments. This illustrated that the ectopic expression of CYP19 dominated the pool.

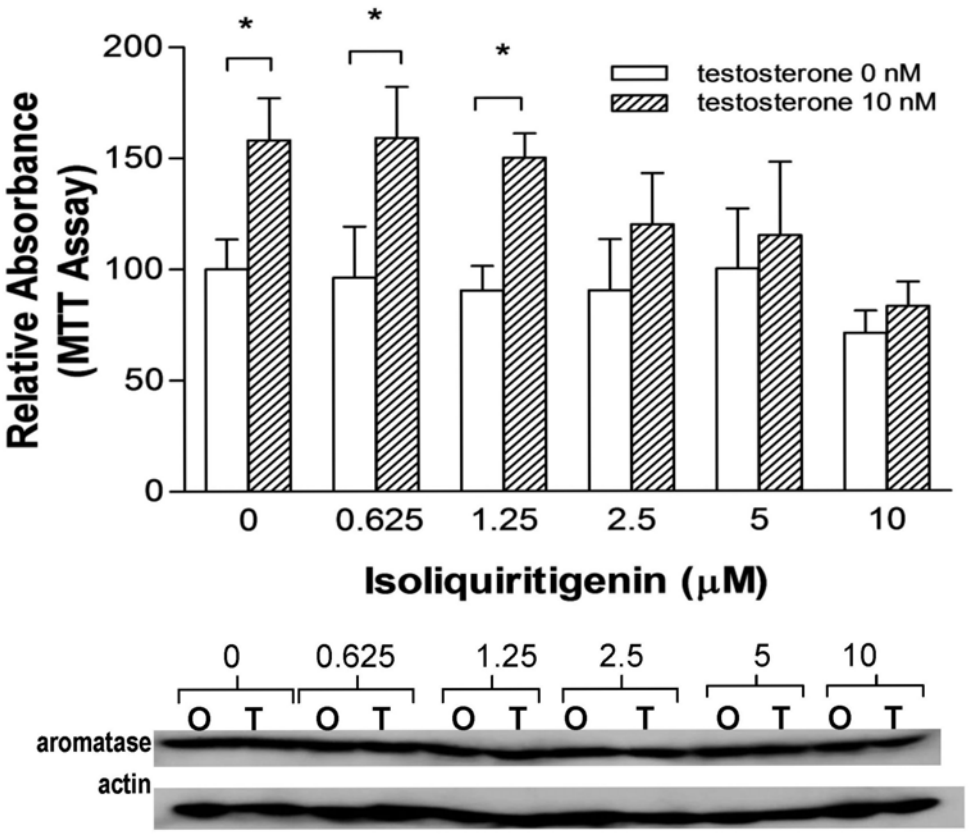



**Fig.4.3** Isoliquiritigenin suppressed MCF-7aro cell proliferation induced by testosterone. MCF-7aro cells were seeded in 96-well plates at $10^4$ cells per well and maintained in medium supplemented with 10% charcoal dextran-treated serum. The effect of isoliquiritigenin on the cell proliferation of testosterone-treated MCF-7aro cells was determined after 48 hrs of incubation. (*) indicates that two means across different testosterone treatment differ ($p < 0.05$), n=6. One-way ANOVA followed by Bonferroni's Multiple Comparison Test if significant differences ($p < 0.05$) were observed. The corresponding protein expression of aromatase is shown in the lower panel; lanes labeled T and O are cells treated with and without 10 nM testosterone, respectively. This result illustrates that ILN inhibited aromatase activity without affecting the aromatase expression.

### 4.2.4 ILN suppressed MCF-7aro xenograft growth in nude mice

The diet treatments did not demonstrate adverse effect on the mice. The body weights of all mice were gradually increased and no significant differences were observed among the four groups (**Fig.4.4)**. On the other hand, tumor sizes of the implant appeared to proliferate throughout the time span of experiment as shown in **Fig.4.5**. The mean tumor volume was 163 ± 11 $mm^3$ in Control mice at Day 77 after inoculation, which doubled the mean tumor size of mice feeding on 5000ppm or 0.5% ILN diet. Significant ($P<0.05$) difference in tumor size between the two groups was observed starting from Day 58 till sacrifice. Mice fed with 500 and 1500ppm ILN also exhibited smaller tumor volume than the control mice starting from Day 68 after implantation and onwards ($P<0.05$). No difference in tumor volume among the mice with ILN treatment was revealed.

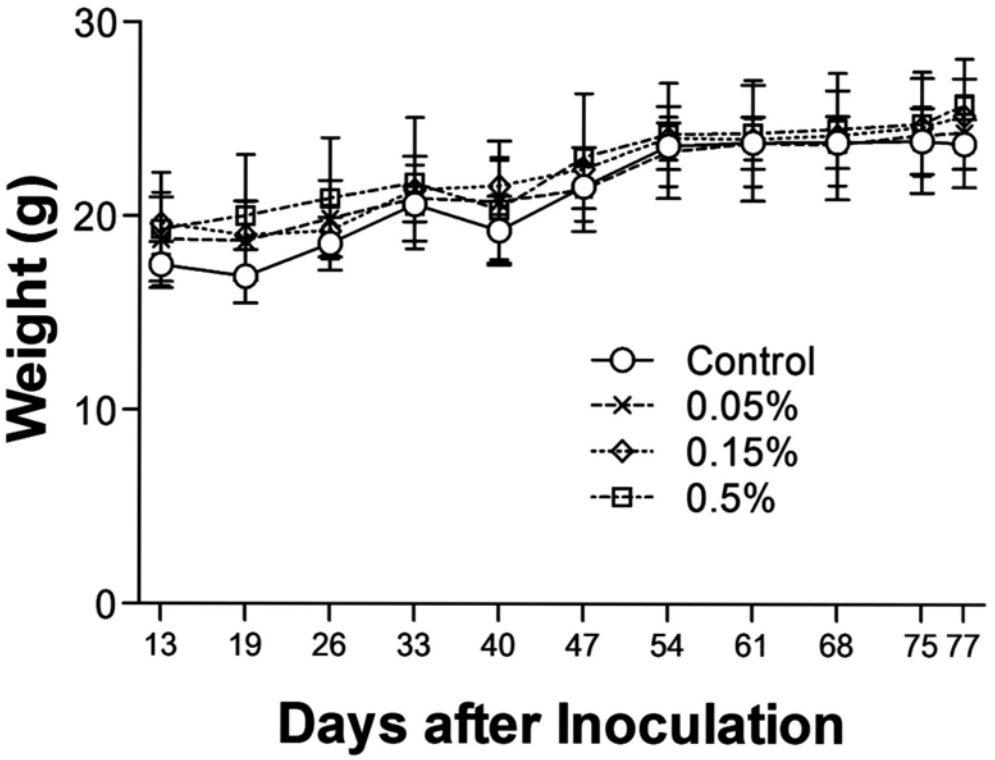



**Fig.4.4** Effect of Isoliquiritigenin on nude mice's body weight. Mice were inoculated with MCF-7aro cells and treated with isoliquiritigenin and androstenedione. Their body weights were monitored from the second week after inoculation. Values are means ± SEMs, n=7 to 10. The data was analyzed by One-way ANOVA, followed by Tukey's Multiple Comparison test.

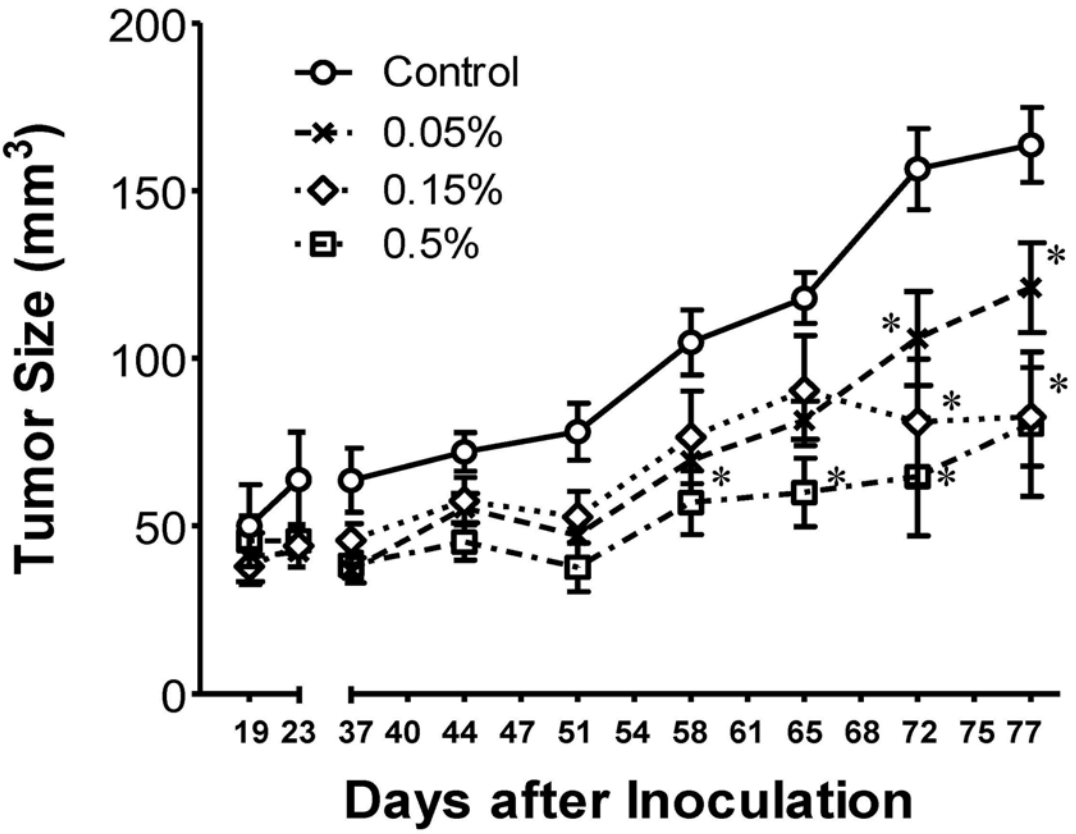



**Fig.4.5** Isoliquiritigenin deterred growth of MCF-7aro transplant tumor in ovariectomized nude mice. Mice were inoculated with MCF-7aro cells at 2 sites per mouse after ovariectomy. They were fed 0, 500, 1500 and 5000ppm isoliquiritigenin in diet and androstenedione was injected *s.c.* every other day starting on the next day after inoculation. Tumor volumes were estimated two times a week from Day 19 after inoculation. Values are means ± SEM, n=7 to 10. The data was analyzed by One-way ANOVA, followed by Tukey's Multiple Comparison test. Means labeled with different letters are significantly different.

### 4.2.5 ILN reduced aromatase mRNA expression in parental MCF-7 cells

MCF-7 cells are commonly used breast cancer model for investigating the effects of phytoestrogens on the disease. As promoters I.3 and II dominate the CYP19 transcriptional regulation, we quantified the mRNA expressions dictated by these promoters. ILN at 10 and 20 μM significantly reduced the exons I.3 and II-spliced transcripts of aromatase, and the results were consistent with the total mRNA expression. The decreases appeared to be in a dose-dependent manner (**Fig.4.6**).

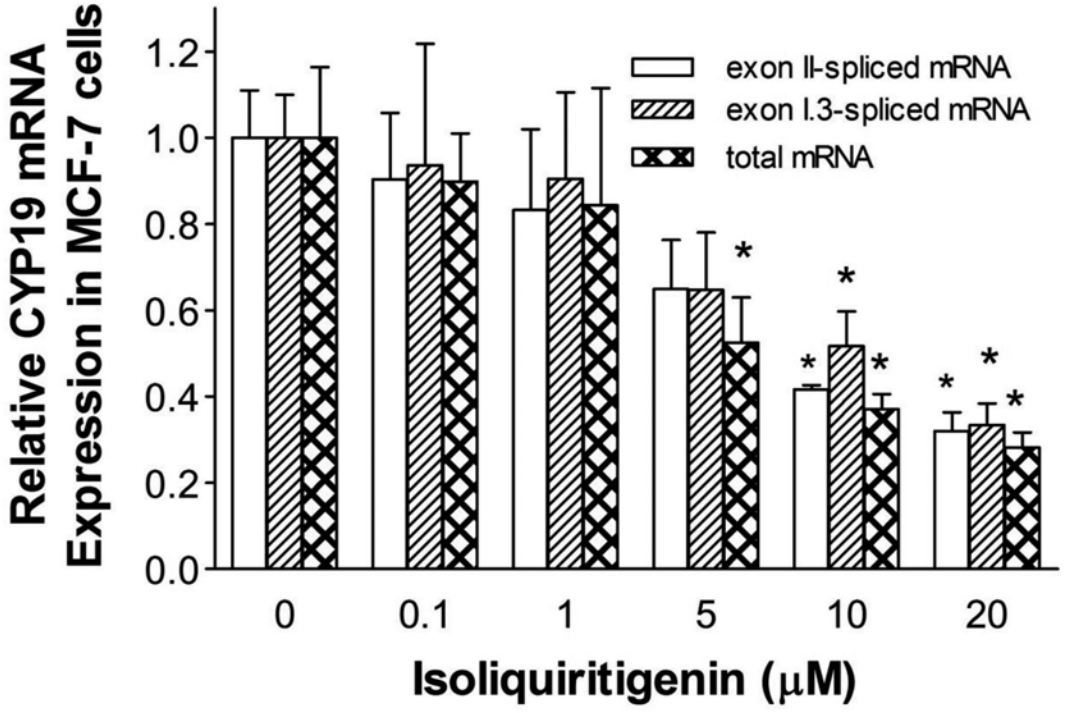



**Fig.4.6** Exon-specific mRNA expression profile under isoliquiritigenin treatment in MCF-7 cells. MCF-7 cells were seeded in six-well plates and maintained in phenol red-free RPMI medium supplemented with 10% charcoal dextran–treated serum. Fig.4.6 profiled the mRNA species derived from different exons expressed in wild-type MCF-7 cells. The suppressive effect of isoliquiritigenin on PI.3 and PII-directed mRNA expression was demonstrated. The exon-specific CYP19 expression was determined by real-time RT-PCR, and the expression was normalized with GAPDH. Values are means ± SEMs, n = 3; and samples are isolated from independent cultures. *Mean values were significantly ($p < 0.05$) different from those of the control cultures with no isoliquiritigenin treatment.

### 4.2.6 Effect of ILN on promoter I.3/II activity of *CYP19* in MCF-7 cells

As the enzyme activity of CYP19 was reduced by ILN, we subsequently determined the transcriptional activity driven by promoter regions I.3 and II. We employed the breast cancer cell line MCF-7, which had been demonstrated using promoter I.3/II for CYP19 regulation (Wang *et al.* 2008), for the assessment of promoter activity. ILN at a concentration of 10 μM or above was able to repress the promoter activity (**Fig.4.7**) ($P<0.05$).

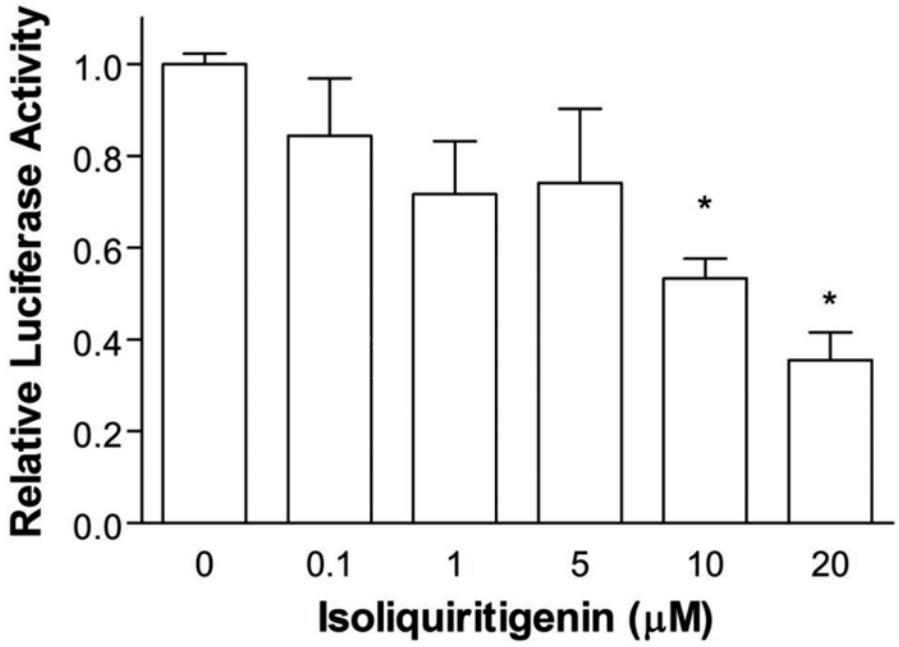



**Fig.4.7** Reporter gene assay of promoter I.3/II in MCF-7 cells. MCF-7 cells were seeded in 24-well plates. After 24 h, the cells were transiently transfected with 0.25ng of the CYP19 reporter plasmid and 2.0ng of renilla luciferase control plasmid. The activities of the luciferases were determined in the cell lysate under a 24-h isoliquiritigenin treatment. One-way ANOVA followed by Bonferroni's Multiple Comparison Test if significant differences ($p < 0.05$) were observed. Values are means ± SEM, n=3. *Means were significantly ($p < 0.05$) different from that of the control.

### 4.2.7 Identification Sequences Responsible For Reduction of Promoter 1.3/II by ILN

ILN could repress promoter 1.3/II activity in MCF-7 cells. To locate the sequence responsible for the reduction, a series of truncate promoters were prepared of promoter 1.3/II and were inserted into the reporter plasmid pGL-basic. Comparing to those transfected with the immediate shorter constructs, cells transfected with (-56/+118) - and (-356/+118) - driven reporter plasmids displayed reduced luciferase activities (**Fig.4.8**). This revealed that some transcriptional factors might be suppressed within (-56/0) and (-356/-306). Since these two sequences also fell in the promoters PII and PI.3 respectively, the reduced transactivity might explain the suppression on exons II and I.3 specific mRNA species in **Fig.4.6**.

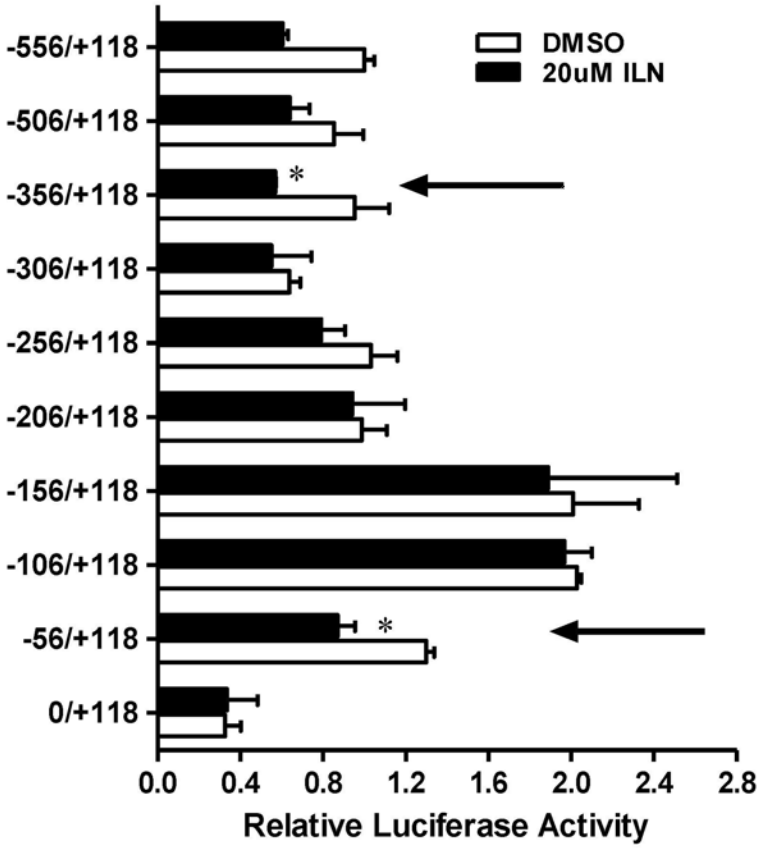



**Fig.4.8** Transcriptional activity in MCF-7 cells transfected with a series of 5'deletion constructs of promoter 1.3/II. 20μM of isoliquiritigenin was used to treat MCF-7 cells transfected with different truncated reporter plasmid for 24 h and the cells were lysed and dual luciferase assay were performed. One-way ANOVA followed by Bonferroni's Multiple Comparison Test if significant differences ($p < 0.05$) were observed. Values are means ± SEM, n=3. *Means were significantly ($p < 0.05$) different from that of the control.

### 4.2.8 ILN reduced C/EBP binding in promoter II DNA fragment

By scanning through the segments screened out from the promoter truncation assay using the software MATCH™, C/EBP binding sites were identified in the two segments (-56/0 and -356/-306) isolated in the above truncation reporter gene assay. Subsequently, EMSA assay also verified that the binding of C/EBP was diminished by ILN dose-dependently (**Fig. 4.9**). The specificity of the band was demonstrated by the disappearance of intensity upon incubating with the antibody of C/EBP.

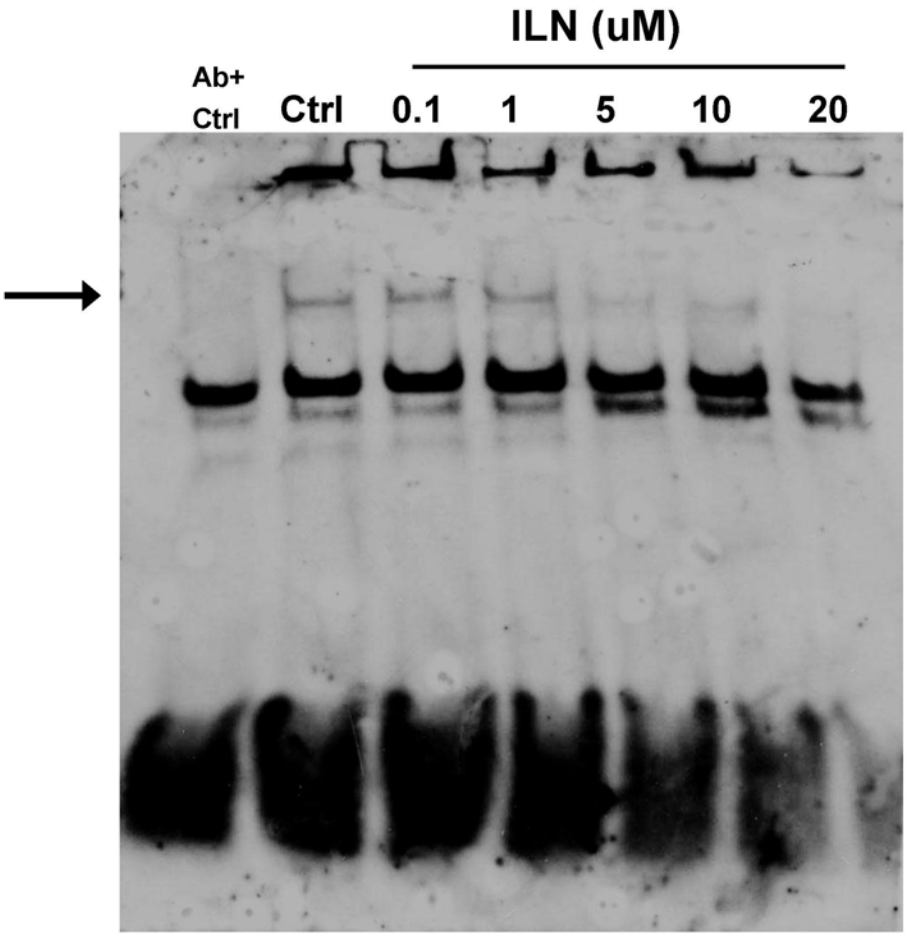
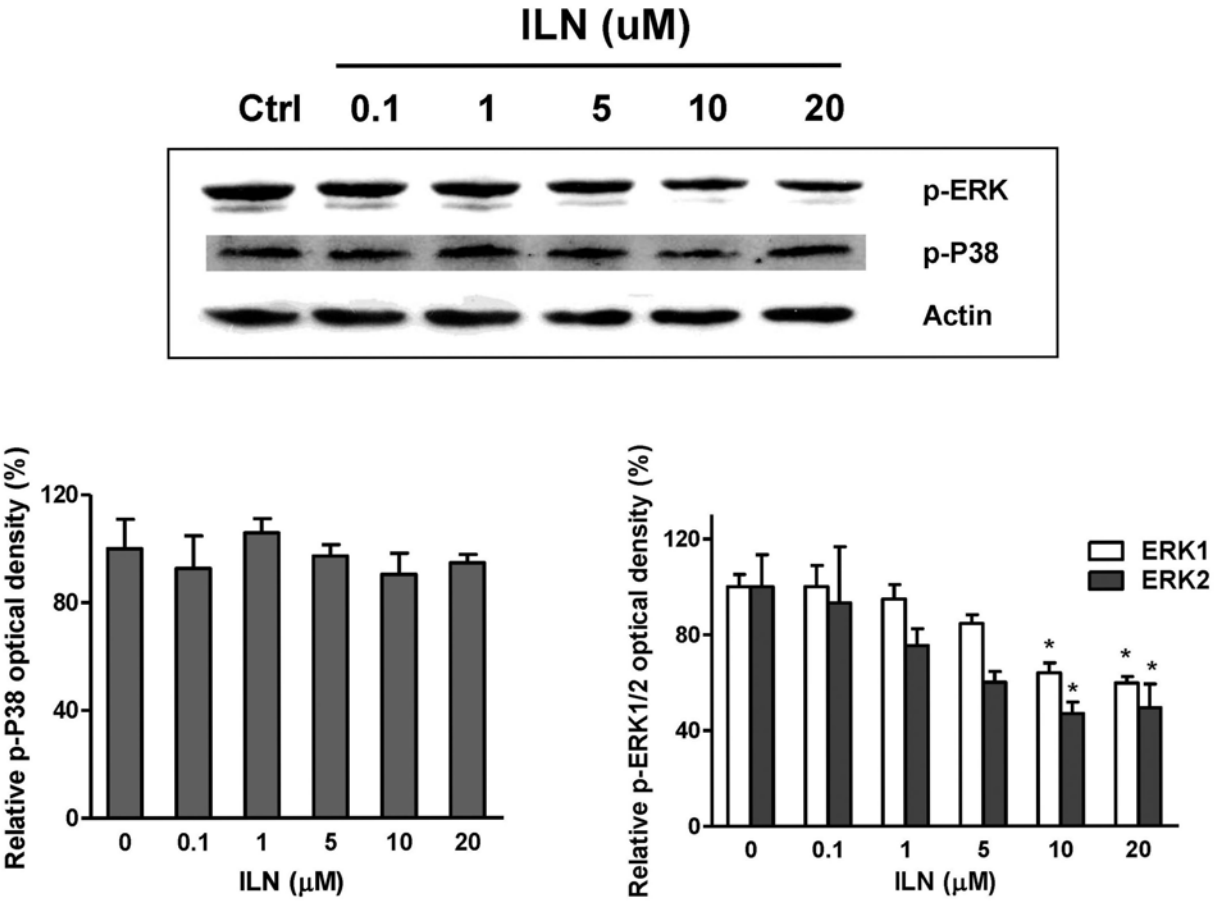



**Fig.4.9** Isoliquiritigenin treatment reduced the electromobility shift of C/EBP. MCF-7 cells were cultured in T-75 flasks and treated with different concentrations of isoliquiritigenin. Nuclear extracts were isolated and used for electromobility shift assays. Fig.4.9 illustrates the band shift detected from extracts incubating with DIG-labeled PII (-47/-32) oligonucleotide; lane AB+Ctrl, C/EBP antibody and control extract; lane Ctrl, control; lanes 0.1, 1, 5, 10, 20, are the concentrations of isoliquiritigenin, respectively. The autoradiograph is a representation of three independent experiments. (➡), C/EBP binding band.

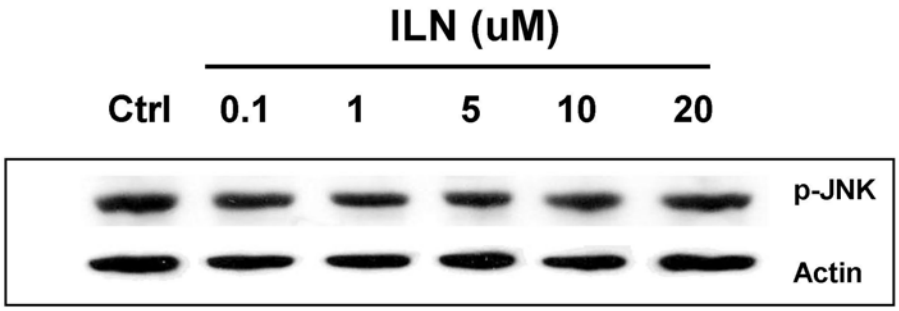
### 4.2.9 Inhibitory effect of ILN on signaling protein kinases

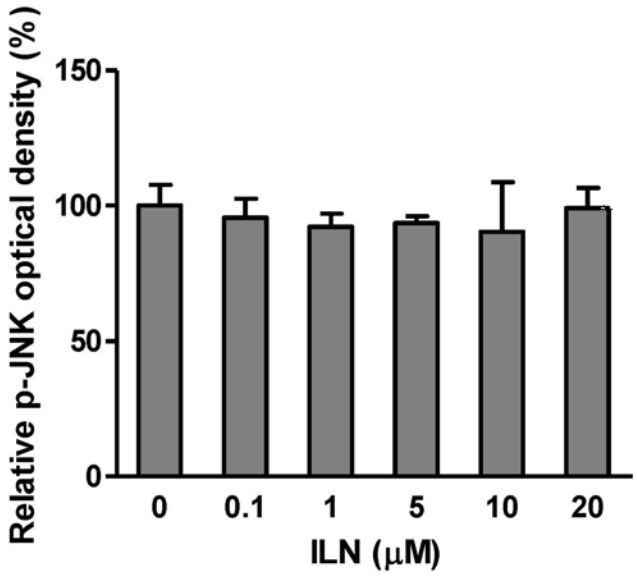
As C/EBP could be activated by phosphorylation, several signaling protein kinases were examined. Western analysis indicated that ILN reduced the phosphorylated forms of MEK and ERK without affecting the amounts of JNK, P38 (**Fig.4.10a-10d**). The deactivation of ERK, which could be the consequence of reduced active MEK, would reduce the C/EBP-driven transactivation of CYP19.

a

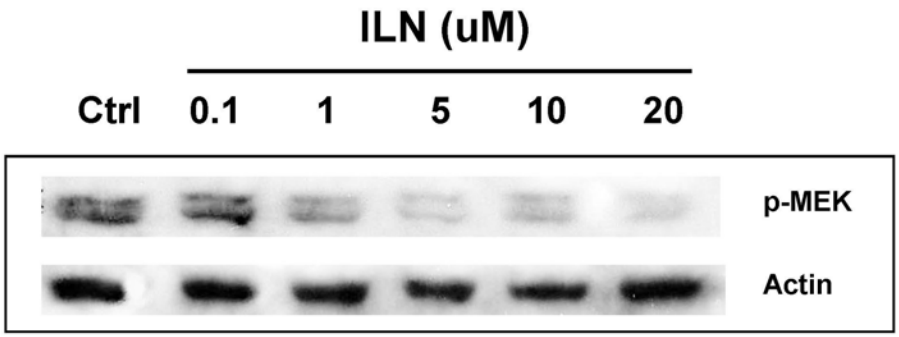


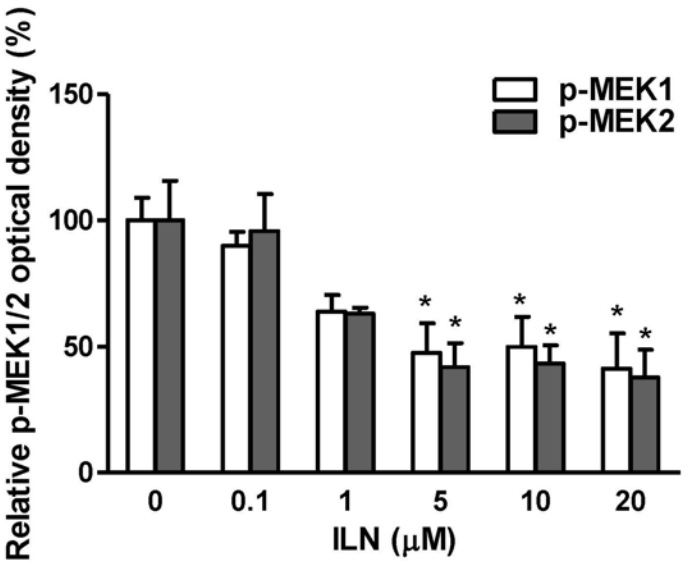
b


ILN (uM)
Ctrl
0.1
1
5
10
20
p-JNK
Actin


Relative p-JNK optical density (%)
150
100
50
0
0
0.1
1
5
10
20
ILN (μM)

C


ILN (uM)
Ctrl
0.1
1
5
10
20
p-MEK
Actin


Relative p-MEK1/2 optical density (%)
150
100
50
0
p-MEK1
p-MEK2
0
0.1
1
5
10
20
ILN (μM)

d

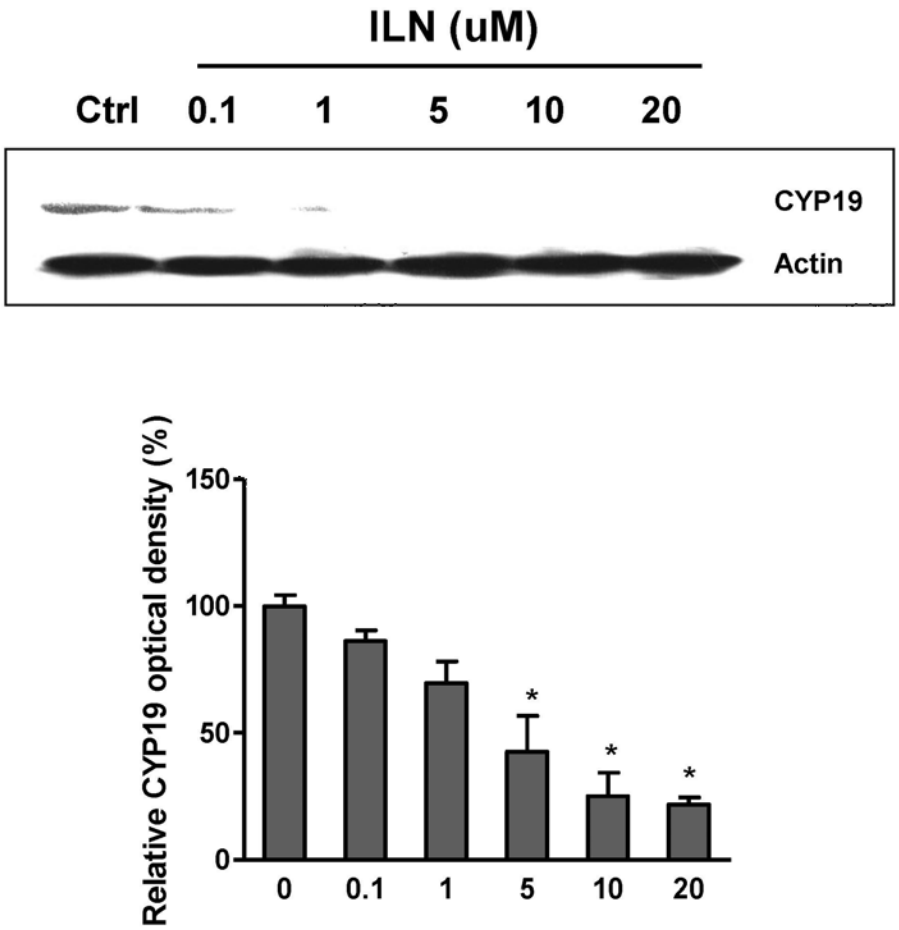



**Fig.4.10** Immunoblot of aromatase and signaling protein kinases in MCF-7 cells treated with isoliquiritigenin. MCF-7 cells were seeded in 6-well culture dishes and treated with isoliquiritigenin for 24 h. Protein expression of phosphoylated forms of ERK-1/2 and P38 (a), JNK (b), MEK-1/2 (c) and CYP19 (d) in cell lysates was determined by western blot analysis. The image is a representation of two independent experiments with similar results. The corresponding optical density readings are shown below the image. There were triplicates of samples in each experiment. The image is a representation of 2 independent experiments with similar results. *Means were significantly ($p < 0.05$) different from that of the control. One-way ANOVA followed by Bonferroni's Multiple Comparison Test if significant differences ($p < 0.05$) were observed.

## 4.3 DISCUSSION

In the first part of the present study, we illustrated that ILN inhibited aromatase at the enzymatic level and a combination of both competitive and non-competitive inhibitions were implicated. ILN could also suppress MCF-7aro cell proliferation attributed to the overexpressing aromatase activity. When MCF-7aro cells were transplanted into ovariectomized nude mice, ILN could also suppress growth of this xenograft. Because this system is widely accepted as a model for post-menopausal breast cancer study, ILN might be a potential therapeutic or chemopreventive natural product for treating this disease.

Other phytochemicals have also been reported to be aromatase inhibitors. Extract of red wine inhibits aromatase activity, and reduces mammary hyperplasia in transgenic mice over-expressing CYP19 (Eng *et al.* 2001). The active ingredients in the extract can be procyanidin B dimmers (Eng *et al.* 2003) and resveratrol (Wang *et al.* 2006). Some flavonoids, like chalcones (Le Bail *et al.* 2001) and biochanin A (Wang *et al.*, 2008), also display inhibitory actions on aromatase. In the present study the flavonoid ILN was demonstrated to inhibit the enzyme activity with a relatively strong potency. Since it has more hydroxyl substituted groups than the other aromatase-inhibitory flavonoids, these groups may alter the microenvironment of the active site by generating steric hindrance or hydrogen bonds.

In the second part of this study, the possibility of ILN on the suppression of CYP19 expression was addressed. At the transcriptional level, the phytocompound could also reduce the aromatase mRNA expression in wide-type MCF-7 cells. The

promoter utilization of human aromatase gene is tissue-specific and promoters I.3 and II (Chen *et al.* 1999) have been identified to be responsible for the expression in breast cancer cells. ILN also suppressed the transactivation dictated by these promoters in MCF-7 cells. This attenuation of CYP19 expression is shared by resveratrol (Wang *et al.* 2006) and biochanin A (Wang *et al.*. 2008 ), though ILN appears to be more potent. Many transcriptional factors or pathways have been described for the regulation of aromatase expression. Simpson *et al* (1997) have reviewed that cyclic AMP, phorbol esters, dexamethasone, prostaglandin (PG) E2, transforming growth factor-β, and γ-interferon increase the transcriptional activity, whereas cyclooxygenase inhibitors suppress the mRNA expression (Diaz-Cruz *et al.* 2005). Given the fact that ILN is a COX-2 inhibitor (Takahashi *et al.* 2004), the CYP19 transcription in the current study can be attenuated through cyclooxygenase inhibition. Further investigation demonstrated that the binding of C/EBP to a DNA sequence within PII promoter of CYP19 was suppressed by ILN.

The CCAAT/enhancer binding proteins (C/EBPs) are a family of transcriptional factors associated with the proliferation and differentiation of various tissues. Its isoforms have been linked with the development of breast cancer (Grimm and Rosen 2003). In addition, the delta isoform of C/EBP can act on PI.3/II and upregulate aromatase expression in breast cancer cells (Kijima *et al.* 2008). In the present study, we identified that C/EBP was deactivated by ILN and the weakened binding to a PII segment might be responsible for the downregulation of CYP19. This deactivation

might be introduced through reducing active MEK and ERK, since ERK was able to phosphorylate and activate C/EBP (Li *et al.* 2007).

Licorice extract has been reported to be antiproliferative to cancer cells *in vitro* (Jo *et al.* 2004; Jo *et al.* 2005). The ethanol extract may induce apoptosis through upregulating the expressions of p53 and Bax. It may also induce G1 cell cycle arrest by increasing the expression of p21, downregulating cdk2 and cyclin E. As a licorice ingredient, ILN can also induce apoptosis and cell cycle arrest in various cancer cell types. Many possible anticancer mechanisms have been described, for instance, reducing the production of PGE2, nitric oxide (Takahashi *et al.* 2006) and ErbB3 (Jung *et al.* 2006), and upregulating p21 expression (Ii *et al.* 2004). Results of the present study provided another possible chemopreventive function for the phytochemical. Similar to other flavonoids, ILN is a weak ER agonist (Maggiolini *et al.* 2002) that may induce cell growth. However, its stimulatory effect on growth was not demonstrated *in vitro* or *in vivo* in MCF-7aro cells.

The transplant model for postmenopausal breast cancer employed in this study has been established and characterized by Yue *et al.* (1994). We demonstrated that ILN could suppress growth of these aromatase-overexpressing tumor cells in nude mice, while the body and liver (data not shown) weights did not significantly deviate from the control. Since other pharmacological properties have been documented as discussed in Introduction for this compound, the suppressive effect on xenograft growth could be the interplay between aromatase inhibition and other biological activities.

In summary, the present study suggested that ILN inhibited the enzyme and transcriptional activity of CYP19. The inhibition was further verified in an animal model. These results suggested that the licorice flavonoid ILN is a potential chemopreventive agent for post-menopausal women against breast carcinogenesis.

# CHAPTER 5
# EFFECTS OF HESPERETIN ON MCF-7aro XENOGRAFT GROWTH IN A POSTMENOPAUSAL MODEL

## 5.1 Introduction

The flavonone 3′,5,7-trihydroxy-4-methoxyflavanone (HES), a member of the flavonone, is found abundantly in citrus fruits (Choi 2007). Citrus fruits have been shown to have antioxidant, antiproliferative and antimetastatic properties (Manthey and Guthrie 2002; Gao *et al.* 2006; Benavente-Garcia *et al.* 2007). Citrus juice consumption can inhibit mammary tumorigenesis induced by 7,12-dimethylbenz[a]anthracene (DMBA) in female Sprague-Dawley rats (So *et al.* 1996; Guthrie and Carroll 1998). In vitro study also has demonstrated that HES inhibited cell proliferation of breast cancer cells and induced G1-phase cell cycle arrest (Choi 2007). Previous studies also shown that HES had potential anticarcinogenic activity in mES cells and exerted chemopreventive effects against 1,2-dimethyl hydrazine induced colon carcinogenesis in rats (Choi *et al.* 2006; Aranganathan *et al.* 2008).

The absorption, metabolism and cellular uptake of citrus flavonoids have been investigated in recent years (Spencer *et al.* 2004).Glycoside form of citrus flavonoids are first de-conjugated by the action of the enzyme β-glucuronidases in the small intestine into the aglycone form (Erlund *et al.* 2001).Together with the originally aglycone form, citrus flavonoids are absorbed by the small intestine and

subsequently transported to different parts of the body through the blood circulation (Spencer *et al.* 2004).The estimated peak plasma concentrations in individuals after consuming orange juice (8 ml/kg) are about 2.2 μM (Erlund *et al.* 2001).

Since HES has been shown to be an aromatase inhibitor in vitro (Jeong *et al.* 1999), the present study is to investigate the potential effects of HES on growth of CYP19-overexpressing MCF-7 (MCF-7aro) cells in ovariectomized nude mice. The animal design was carried out as follows:

**Animal model** This study addressed whether the flavonone HES can act as a chemopreventive agent. The protocol of this model for postmenopausal breast cancer was performed as described by Yue *et al.* Thirty female nude mice aged around 6 weeks were acquired from Animal Facility of Chinese University of Hong Kong. The mice were ovariectomized and allowed 3 weeks to recover. The mice were randomly divided into 5 groups. All the groups were transplanted with MCF-7aro cells. Before transplantation, the MCF-7aro cells were maintained in culture incubator. After growing to 80% confluency, the cells were trypsinized, suspended in matrigel matrix (10 mg/ml) and 0.1 ml of cells ($3 \times 10^7$ cells/ml) were injected into the two flanks of the back of each animal. 0.1 mg of androstenedione was also injected *s.c.* every other day except for C group throughout the experiment. The dosage of androstenedione has been proved to stimulate the MCF-7aro cell proliferation previously (Yue *et al.* 1994). Mice were fed with one of four regimens: 0, 500, 1000, 5000ppm HES mixed in semipurified AIN-93G diet. The mice body weight and tumor size of the mice were monitored weekly and food intake were

measured through the study. Tumor volumes were measured by an electronic caliper and estimated according to the formula: π /(6×length× width × height), where length, width, and height were the three orthogonal diameters of the tumors. At the end of the study, the mice were sacrificed by cervical dislocation. Tumors, blood, liver and uterus samples were collected for assays.

## 5.2 RESULTS

### 5.2.1 Enzyme inhibition assay performed on MCF-7aro cells

MCF-7aro cells are commonly used for enzyme inhibition analysis which has been shown by previous studies. HES inhibited aromatase activity in MCF-7aro cells with $IC_{50}$ value about 5μM **(Fig.5.1)**.

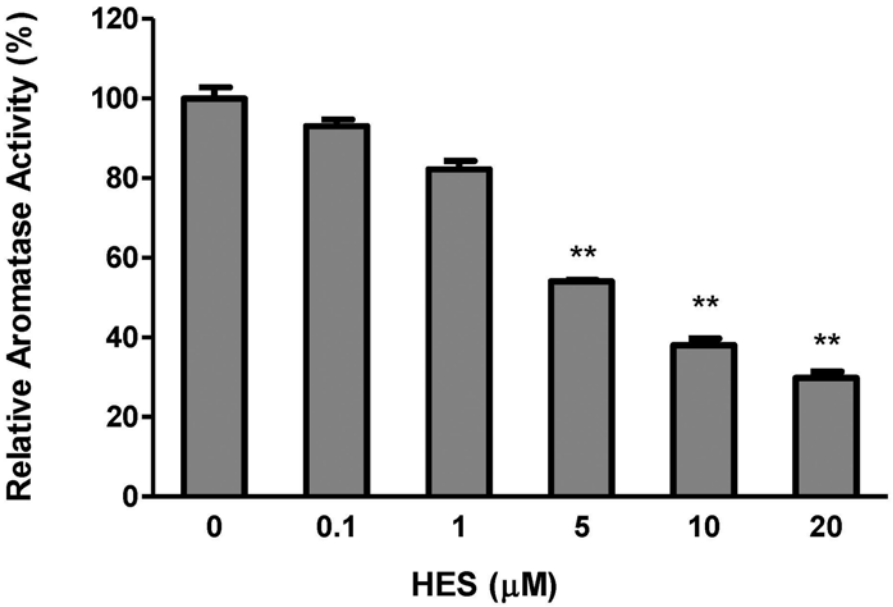



**Fig.5.1 Inhibition of aromatase activity by HES in MCF-7aro cells.** MCF-7aro cells were incubated with 25nM [1β-$^{3}$H(N)]-androst-4-ene-3, 17-dione and co-treated with HES for 1 h. The amount of tritiated water released was measured as described. The $IC_{50}$ values were determined to be about 5μM. One-way ANOVA followed by Bonferroni's Multiple Comparison Test if significant differences ($p < 0.05$) were observed. Values are means ± SEM, n = 3. (** $p<0.01$)

### 5.2.2 Specific inhibition on testosterone-induced proliferation in MCF-7aro cells

HES was able to reduce the testosterone-induced proliferation of MCF-7aro cells through the inhibition of aromatase **(Fig.5.2)**. The administration of 10nM testosterone increased the cell number by 60% as shown at 0μM of HES. At 10μM, HES could bring down the testosterone-induced cell growth to a level comparable to their testosterone-less counterparts.

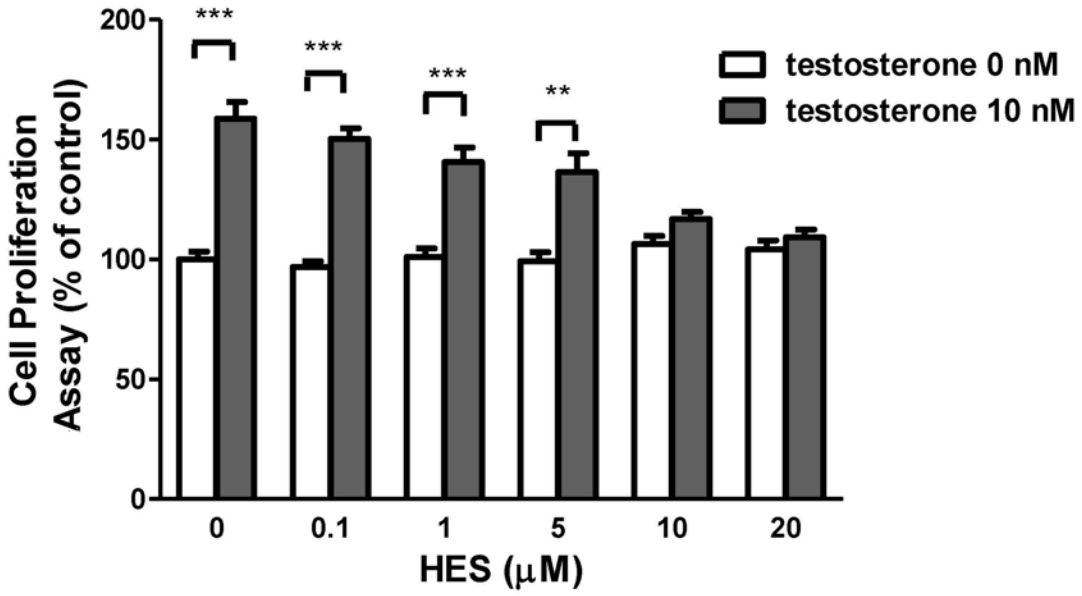
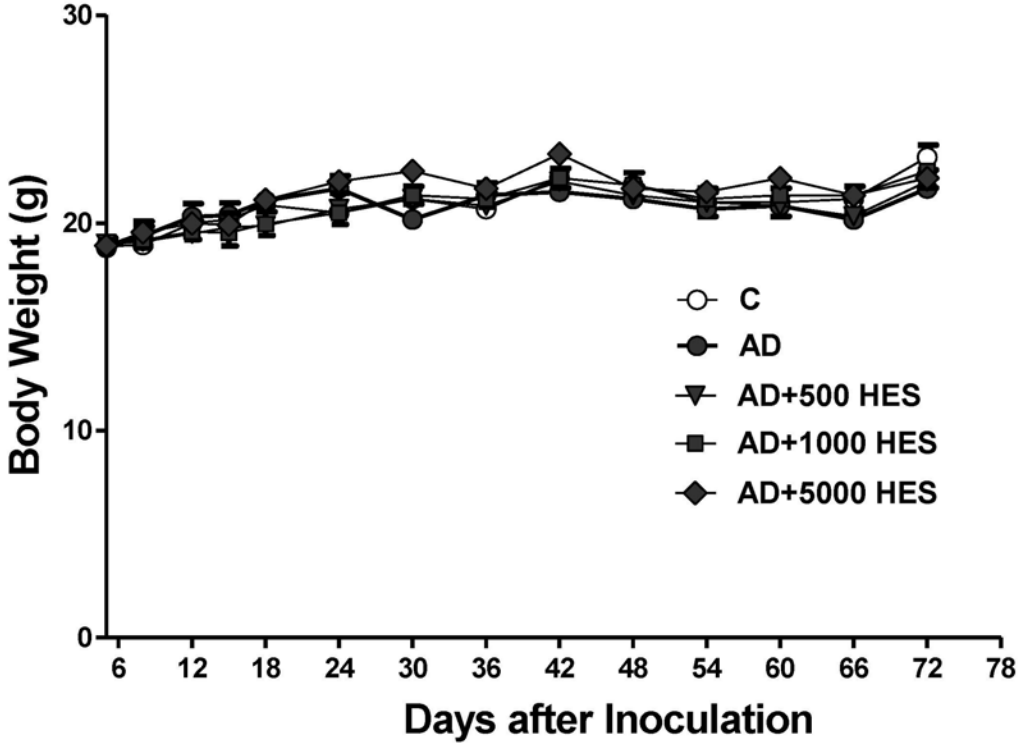



**Fig.5.2. HES suppressed MCF-7aro cell proliferation induced by testosterone.** MCF-7aro cells were seeded in 96-well plates at $10^4$ cells per well and maintained in medium supplemented with 10% charcoal dextran-treated serum. The effect of HES on the cell proliferation of testosterone-treated MCF-7aro cells was determined after 48 hrs of incubation. Values are means ± SEM, n = 8. (** $p<0.01$, *** $p<0.001$)

### 5.2.3 Effect of HES on mouse body and liver weights

The body weights were measured weekly since checking out from the animal facility. The body weights of all mice were gradually increased and no significant differences were observed among the five groups **(Fig.5.3.a)**. There were also no significant differences in the liver weights among the five groups upon sacrifice **(Fig.5.3.b)**. Feed intake was measured throughout the study, and no differences were observed among the treatment groups (data not shown).The injection of androstenedione and diet treatments did not induce adverse effect on the mice.

a



b

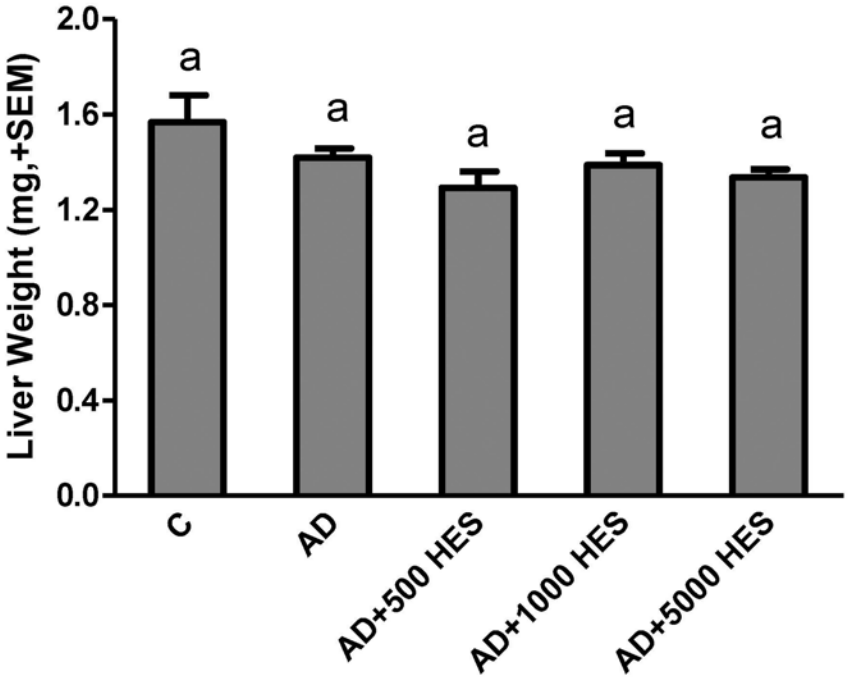
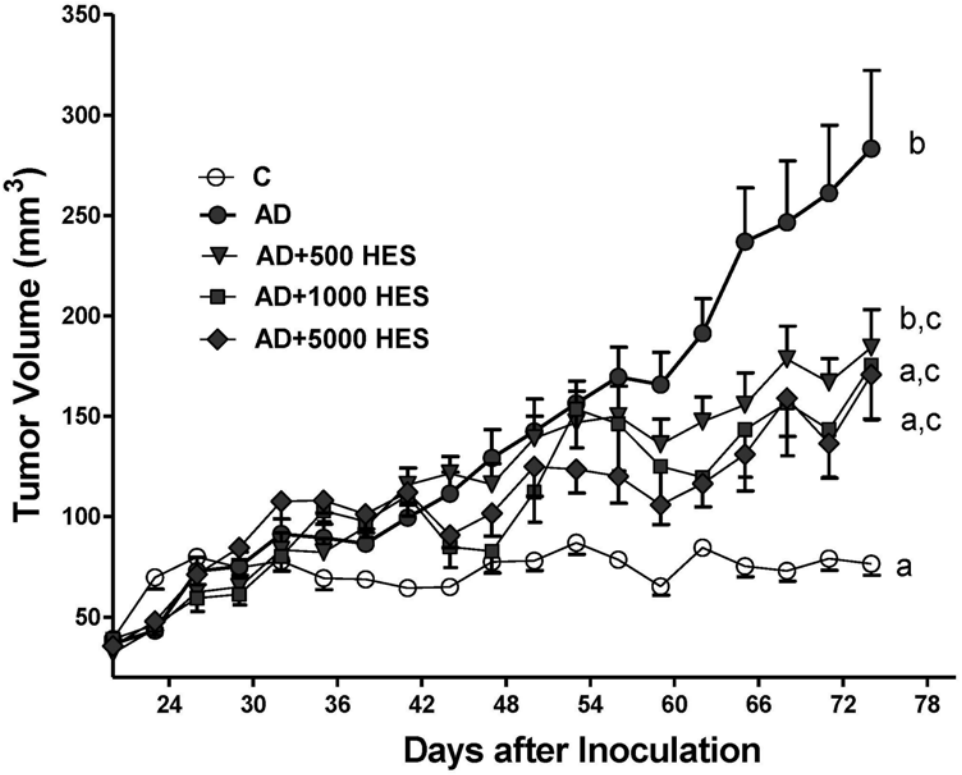



**Fig.5.3 Effect of HES and AD on nude mice's body and liver weight.** Mice were inoculated with MCF-7aro cells and treated with HES. All the groups except group C were injected androstenedione. Their body weights were monitored from the first week after inoculation and the liver weights were measured at the end of experiment. AD-androstenedione,500 HES-500ppm HES, 1000 HES-1000ppm HES, 5000 HES-5000ppm HES. No significant differences were observed among the five groups in body weights (a) and liver weight (b).Values are means ± SEM, The data collected was analyzed by One-way ANOVA, followed by Tukey's Multiple Comparison test.

### 5.2.4 Dietary HES in the presence of AD suppressed MCF-7aro xenograft growth in ovariectomized nude mice

Androstenedione (0.1 mg/mouse) injections s.c. every other day significantly and consistently accelerated the MCF-7aro tumor growth in ovariectomized mice compared with C group which received injection with vehicle **(Fig.5.4a)**.The average tumor volume in the AD group was 99.5 ± 24 at the Day 41, which increased by about 1.5 × over C group. Significant ($P<0.05$) difference in tumor size between these two groups was observed starting from Day 41 till sacrifice. Mice fed with 1000 and 5000ppm HES exhibited smaller tumor volume than the control mice starting from Day 62 after implantation and onwards **(Fig.5.4a)** ($P<0.05$). There were statistically differences between AD group and AD+5000 HES group at Day 65 and 71 while there were no significantly differences observed between the two groups on the other days. The tumor weights measured at the day of sacrifice revealed that there were significant differences between C group and AD group **(Fig.5.4b)** ($P<0.05$). The tumor weights in group AD were heavier than those in group AD+1000 HES and group AD+5000 HES ($P<0.05$), which was consistent with the results from the tumor growth **(Fig.5.4b)**. No difference in tumor volume and tumor weights among the mice with HES treatment was revealed.

a


Tumor Volume (mm3)
Days after Inoculation
C
AD
AD+500 HES
AD+1000 HES
AD+5000 HES
b
b,c
a,c
a,c
a
350
300
250
200
150
100
50
24
30
36
42
48
54
60
66
72
78

b

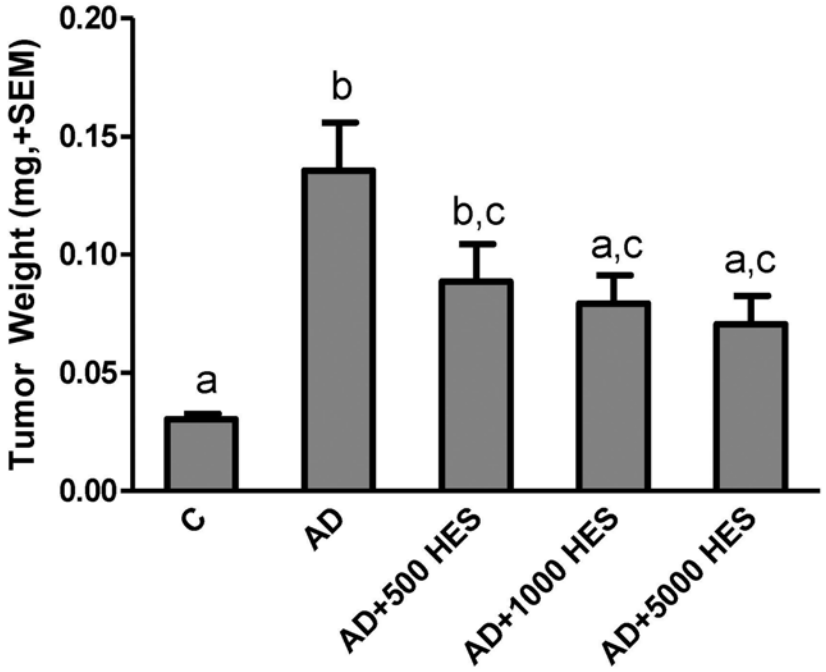



**Fig.5.4 HES deterred growth of MCF-7aro transplant tumor in ovariectomized nude mice.** Mice were inoculated with MCF-7aro cells at 2 sites per mouse after ovariectomy. They were fed 0, 500, 1000 and 5000ppm HES in diet and androstenedione was injected *s.c.* every other day except C group starting on the next day after inoculation. Tumor volumes were estimated two times a week from Day 20 after inoculation (**Fig.5.4a**). The tumor weights were measured at the day of sacrifice (**Fig.5.4b**). Values are means ± SEM, n=11 to 12. The data was analyzed by One-way ANOVA, followed by Tukey's Multiple Comparison test. Means labeled with different letters are significantly different.

### 5.2.5 Plasma E2 levels

Serum collected from group AD displayed a significant increase of estradiol concentration compared with group C **(Fig.5.5)**. This result demonstrated that androstenedione injection could increase serum estrogen concentration. The E2 levels in the AD +1000 HES and AD+5000 HES groups were significantly lower than those in group AD ($p < 0.05$). No significant difference among HES groups and between the C group and the HES groups were detected **(Fig.5.5)**.

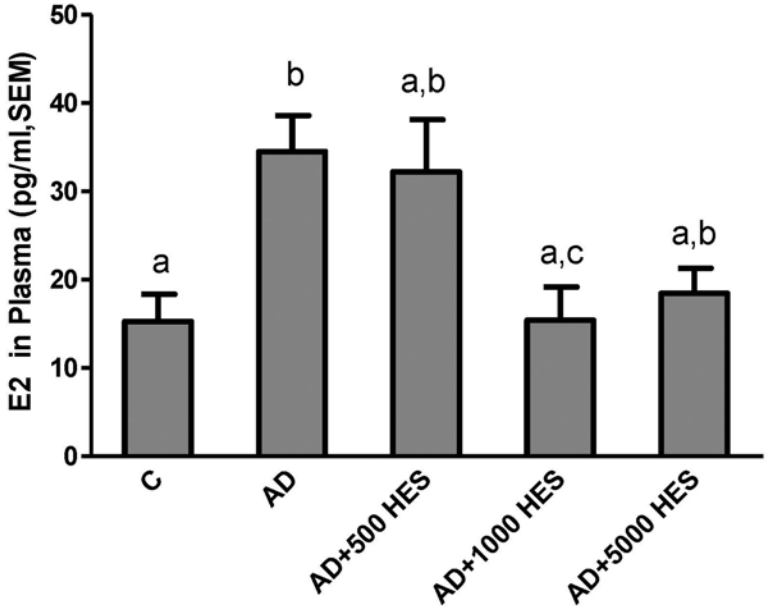



**Fig.5.5. Serum estradiol concentration in ovariectomized mice**. Blood was drawn from the animals at sacrifice. Serum estradiol concentration was quantified by ELISA. Values are means ± SEM, n=6.The data was analyzed by One-way ANOVA, followed by Tukey's Multiple Comparison test. Means labeled with different letter are significantly different.

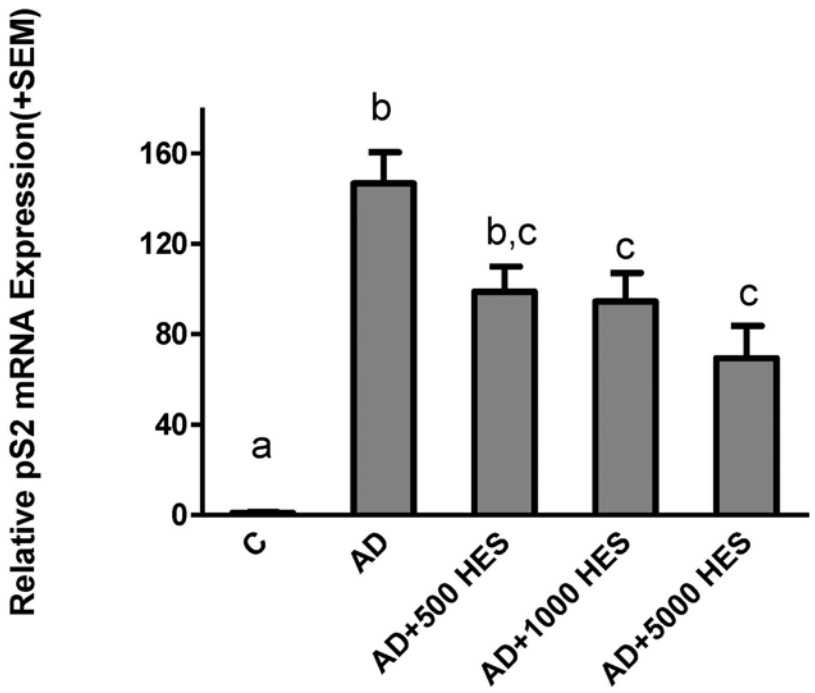
### 5.2.6 E2 responsive and metabolic gene expression in tumors

Total mRNA was extracted and evaluated for the E2 responsive and metabolic gene expression. The following E-responsive genes pS2 and E2 metabolic gene, CYP1A1, CYP1B1, UGT1A1 and COMT, were evaluated by using real-time PCR (**Fig.5.6 a & b**).

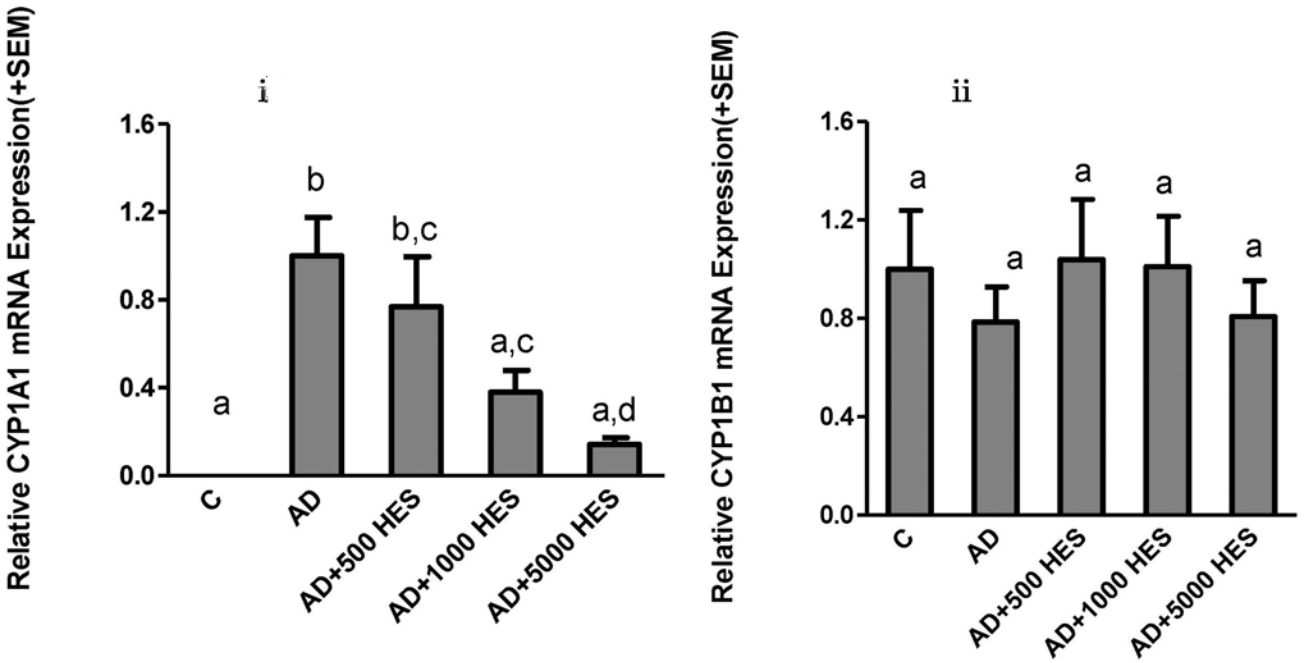
**pS2.** pS2 expression was significantly increased in AD group and the expression was 146 fold higher than that in C group **(Fig.5.6a)**. The addition of dietary HES treatment with 1000ppm and 5000ppm inhibited pS2 expression induced by AD injection ($P < 0.05$).

**CYP1A1, CYP1B1, COMT and UGT1A1.** The CYP1A1 expression was significantly increased in AD group compared with C group ($p<0.05$). The induced CYP1A1 expression was inhibited in AD +1000 HES and AD+5000 HES groups **(Fig.5.6b[ i ])**. The injection of AD significantly increased the CYP1A1 expression while had no effects on CYP1B1 expression. There were no statistically difference in CYP1B1 expression among the five groups **(Fig.5.6B[ ii ])**. The expression of COMT and UGT1A1 was greatly suppressed in AD group **(Fig.5.6B[ iii ], Fig.5.6B[iv])**, however, no statistically differences were observed between AD and HES groups.

a


Relative pS2 mRNA Expression(+SEM)
160
120
80
40
0
a
b
b,c
c
c
C
AD
AD+500 HES
AD+1000 HES
AD+5000 HES

b


Relative CYP1A1 mRNA Expression(+SEM)
i
1.6
1.2
0.8
0.4
0.0
a
b
b,c
a,c
a,d
C
AD
AD+500 HES
AD+1000 HES
AD+5000 HES
Relative CYP1B1 mRNA Expression(+SEM)
ii
1.6
1.2
0.8
0.4
0.0
a
a
a
a
a
C
AD
AD+500 HES
AD+1000 HES
AD+5000 HES

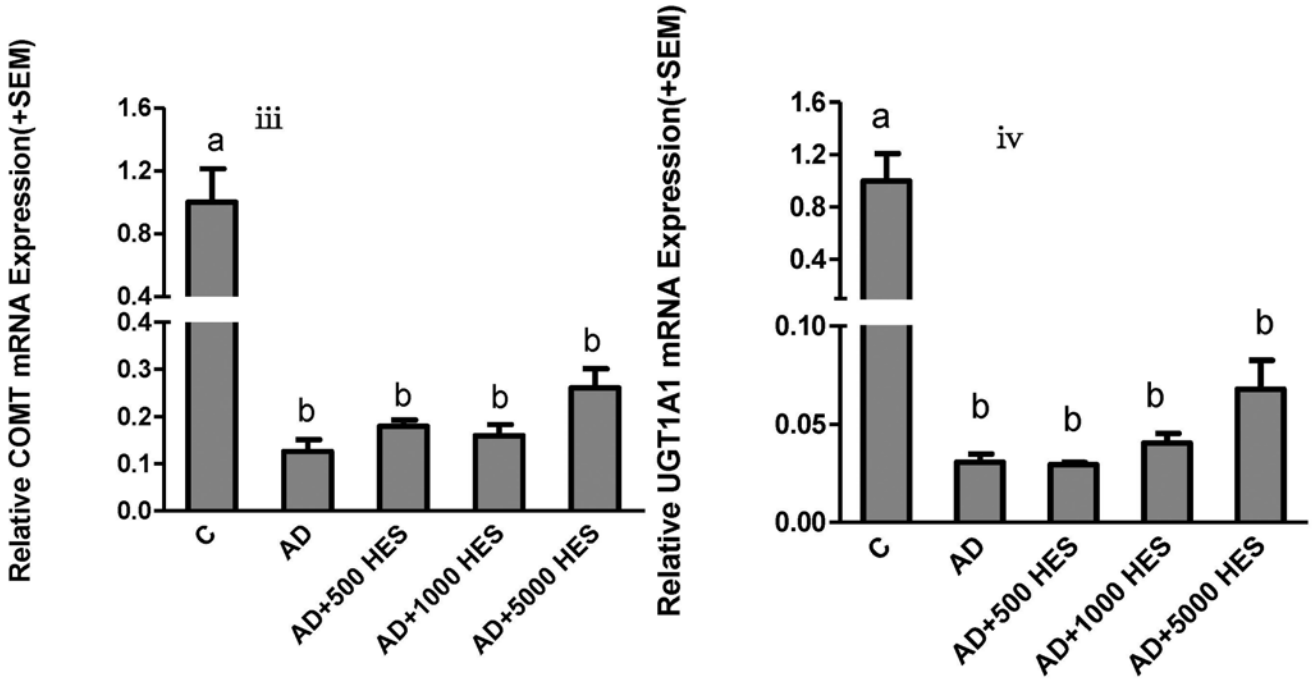


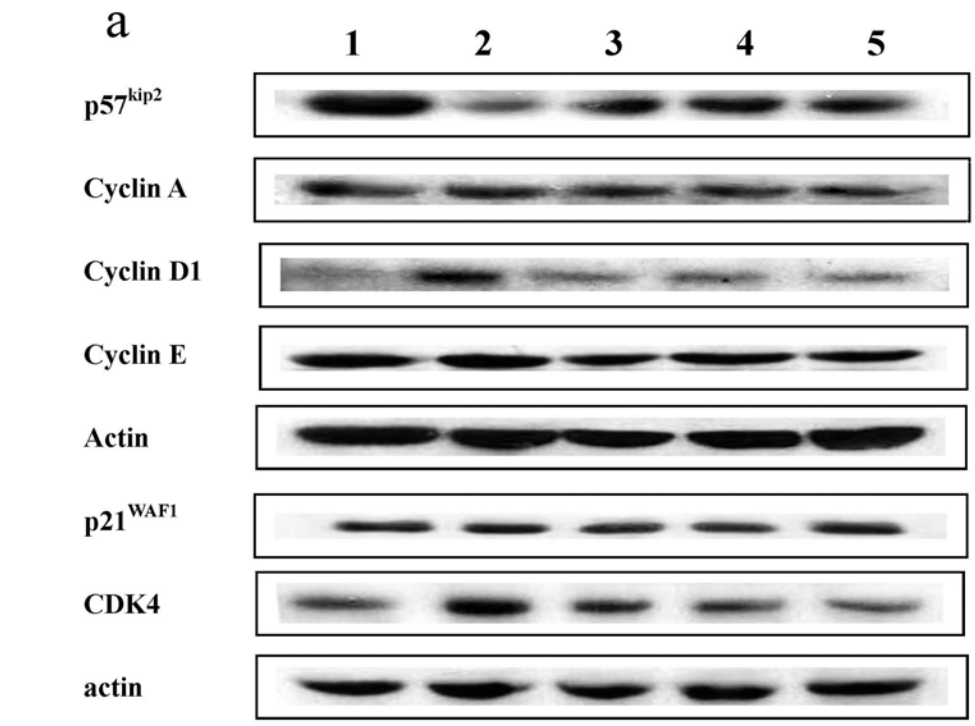

**Fig.5.6. Relative pS2, CYP1A1, CYP1B1, COMT and UGT1A1 mRNA levels in tumors.** Total mRNA was extracted from tumors and messenger RNA expression of these genes was quantified by real-time PCR. Values are means ± SEM, n=5 to 6. The data was analyzed by One-way ANOVA, followed by Tukey's Multiple Comparison test. Means labeled with different letter are significantly different.

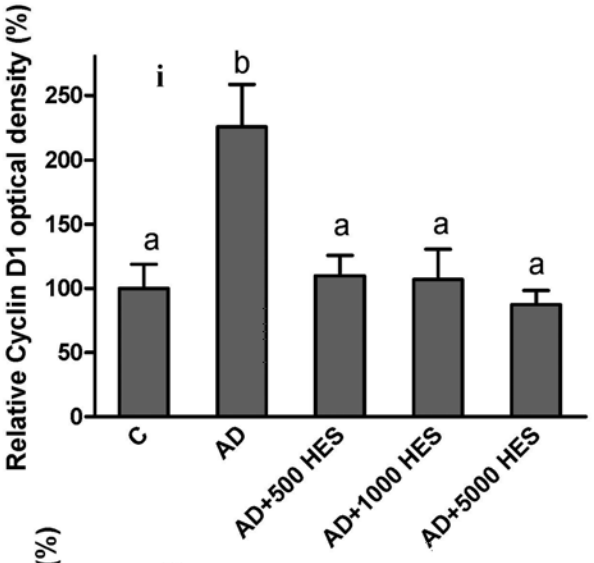
### 5.2.7 Protein expression of cell apoptotic and cell cycle-regulated gene markers

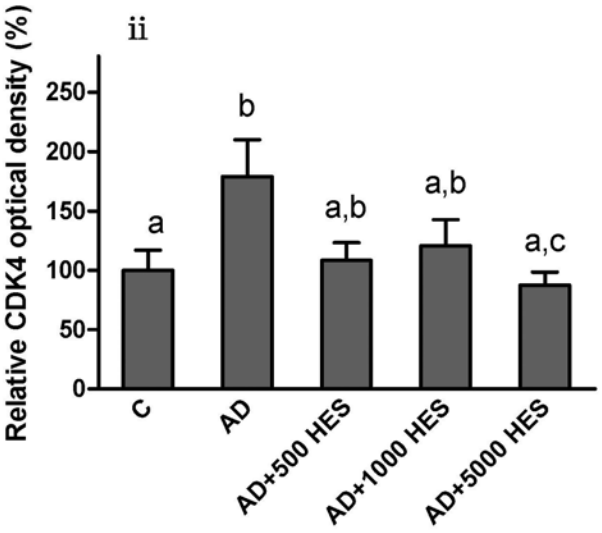
HES has been demonstrated to inhibit cell proliferation and G1-phase cell cycle arrest in MCF-7 cells. As we observed that HES has inhibitory effects on tumor growth in this present study, we further examined the expression of cell apoptotic and cell cycle-regulated gene in vivo.

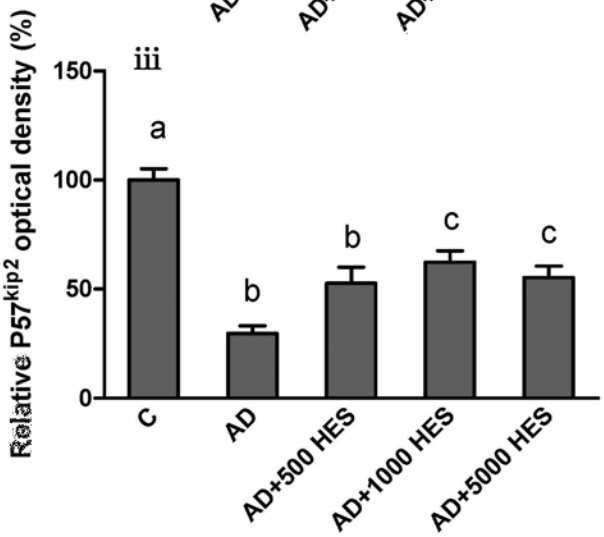
**Cell cycle gene markers.** The cyclin D1 protein content in AD group was higher than that in C group and the induced protein expression was greatly suppressed by HES treatment **(Fig.5.7a)**. The CDK4 protein content was slightly induced by AD injection and the stimulation was counteracted by HES treatment with 5000ppm **(Fig.5.7a)**. The $p57^{kip2}$ protein expression was significantly inhibited by AD injection and HES treatment with 1000ppm and 5000ppm significantly increased the $P57^{kip2}$ protein expression ($P<0.05$). We observed no statistical differences in Cyclin A, Cyclin E and $p21^{WAF1}$ protein expression among the five groups **(Fig.5.7a)**.

**Cell apoptotic gene markers.** Western analysis in **Fig.5.7b** indicated that AD injection induced Bcl-xL expression and HES treatment with 1000ppm and 5000ppm inhibited the induced expression. The protein expression of Bax in AD group was lower than that in C group and HES treatment had no effects on Bax expression. There were no differences in Bcl-2 and Bak protein expression among the five groups **(Fig.5.7b)**.

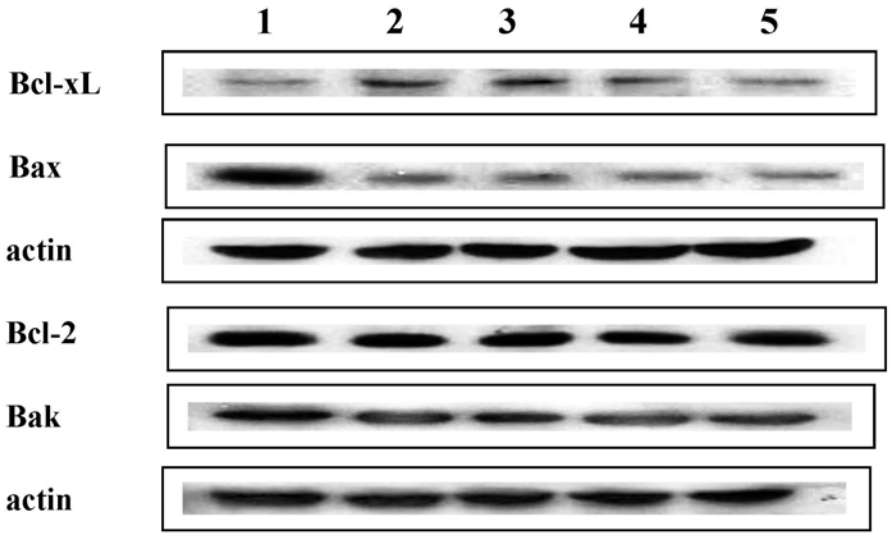

a
1
2
3
4
5
p57kip2
Cyclin A
Cyclin D1
Cyclin E
Actin
p21WAF1
CDK4
actin

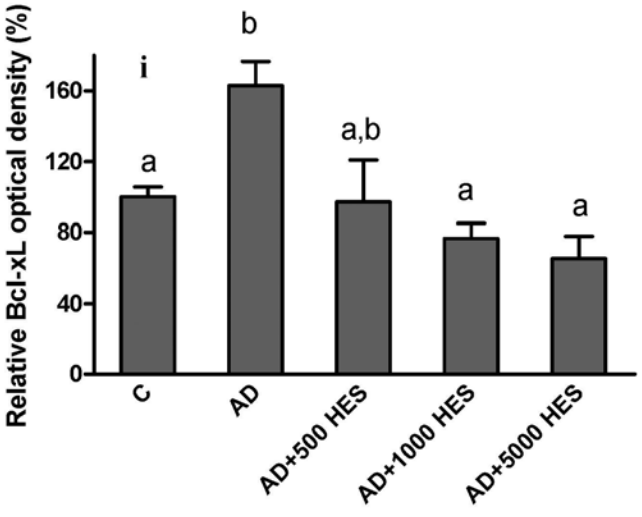

i
Relative Cyclin D1 optical density (%)
250
200
150
100
50
0
a
b
a
a
a
C
AD
AD+500 HES
AD+1000 HES
AD+5000 HES

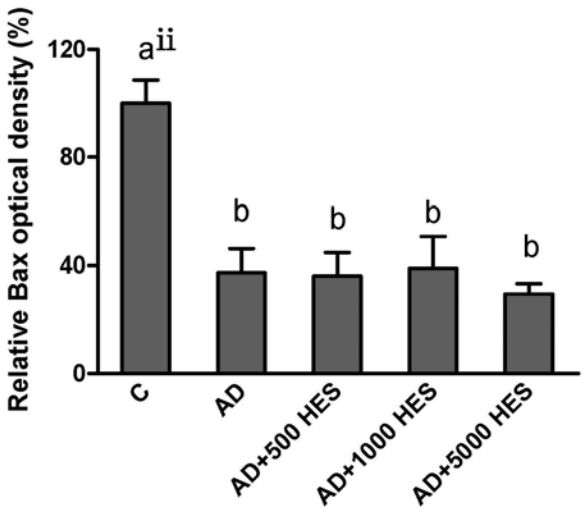

ii
Relative CDK4 optical density (%)
250
200
150
100
50
0
a
b
a,b
a,b
a,c
C
AD
AD+500 HES
AD+1000 HES
AD+5000 HES


iii
Relative P57kip2 optical density (%)
150
100
50
0
a
b
b
c
c
C
AD
AD+500 HES
AD+1000 HES
AD+5000 HES

b


1
2
3
4
5
Bcl-xL
Bax
actin
Bcl-2
Bak
actin


Relative Bcl-xL optical density (%)
0
40
80
120
160
i
a
b
a,b
a
a
C
AD
AD+500 HES
AD+1000 HES
AD+5000 HES


Relative Bax optical density (%)
0
40
80
120
aii
b
b
b
b
C
AD
AD+500 HES
AD+1000 HES
AD+5000 HES

Ratio of Bcl-xl/Bax

| C | AD | AD+500ppm | AD+1000ppm | AD+5000ppm |
|---|---|---|---|---|
| 1 | 4.36 | 2.68 | 1.95 | 2.21 |

**Fig.5.7. Immunoblot of cell apoptotic and cell cycle-regulated gene markers.in tumors.** Protein expression of cell cycle-regulated gene (a) and cell apoptotic gene (b) was determined by western blot analysis. The corresponding optical of density readings are shown in the lower panel. The image is a representation of 2 independent experiments in triplicates with similar results. The data was analyzed by One-way ANOVA, followed by Tukey's Multiple Comparison test. Means labeled with different letter are significantly different.

### 5.2.8 Uterine Wet Weight

AD injection increased the uterus wet by 2.9 times over C group ($p < 0.05$). Dietary HES treatment (500, 1000 and 5000ppm) did not change uterine wet weight in ovariectomized MCF-7aro implanted mice with AD injection. This trend of uterine weight was consistent to the corresponding serum estradiol concentration between C and AD group. There was no statistical difference observed between AD and HES groups **(Fig.5.8)**.This result suggested that HES might have uterotrophic action, which was mediated directly by its interaction with uterine ER, thereby counteracted the effects of eliminated plasma E2 levels on uterus weight in HES groups.

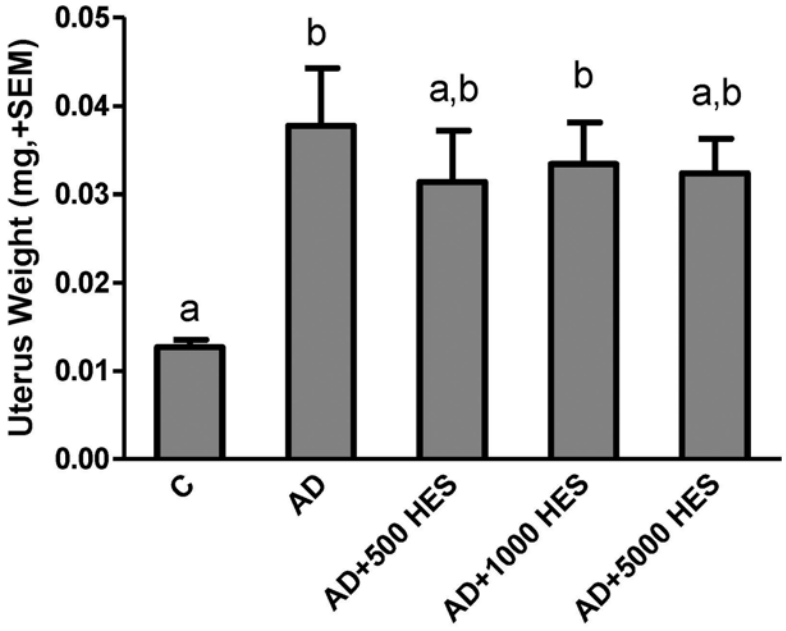



**Fig.5.8. Uterine weight in ovariectomized nude mice.** Uteri of the experimental animals were dissected and weighed at sacrifice. Values are means ± SEM, n=6. The data was analyzed by One-way ANOVA, followed by Tukey's Multiple Comparison test. Means labeled with different letter are significantly different.

## 5.3 Discussion

In the present study, we utilized an ovariectomized nude mouse model to evaluate the effect of dietary HES. HES reduced MCF-7aro cell proliferation pertaining to the inhibition of aromatase activity. When MCF-7aro cells were transplanted into ovariectomized nude mice, AD injection significantly stimulated the xenograft growth and dietary HES administed at 1000ppm and 5000ppm suppressed the induced xenograft growth. AD is a substrate for aromatization and can be converted to E2 by aromatase. The estrogen synthesized stimulated the growth of MCF-7aro cells in vitro and in nude mice (Yue and Brodie 1993). Because this system is widely accepted as a model for post-menopausal breast cancer study, HES might be a potential therapeutic or chemopreventive natural product for treating this disease. An increase in circulating E2 levels was observed in AD-induced mice, and HES treatment at 1000ppm and 5000ppm reduced AD-induced E2 levels. This result suggested that HES could inhibit local aromatase activity in the MCF-7aro tumors. The uterine wet weight result in the current study appeared to be correlated with the serum estradiol concentration. Compared to the control mice, an increase in the uterine weight in the AD group was observed, HES treatment didn't suppress the AD-induced uterine enlargement. HES could be a weak agonist on estrogen receptor, which increased uterus weight. We further evaluated expression of E2 responsive genes and genes responsible for E2 metabolism in the tumors by using the real-time PCR. An indicative to the exposure of estrogenic compound in tissues, pS2 expression was induced in AD mice and dietary HES was able to reverse the induced

expression. This suggested that HES could decrease the situ E2 production by inhibiting the aromatase activity in tumors.

The expression of CYP1A1 was significantly induced in mice treated with androstenedione and co-treatment of AD and HES treatment (1000 and 5000ppm) elicited a suppressive effect of HES on CYP1A1. No alternations of CYP1B1 expression were observed within these treatment groups. Both the UGT1A1 and COMT expression in the tumors were decreased in AD group while HES treatment could not reverse the decrease. CYP1A1 and CYP1B1 are phase I enzymes responsible for hydroxylating estrogen at the position of C-2 and C-4, respectively. These two enzymes have been shown to metabolize xenobiotics and endogenous compounds such as estradiol. The presence of CYP1A1 in some human cancer tissues has been reported (McKay *et al.* 1995; Huang *et al.* 1996). Furthermore, an increased expression of CYP1A1 was found in tissue from breast cancer patients than that from healthy individuals (Goth-Goldstein *et al.* 2000). Present results suggested that the suppression of HES on tumor growth may partially through its inhibition on CYP1A1 expression. This result was also consistent with a previous study, in which hesperetin was estimated to be an inhibitor of human CYP1A1 by ethoxyresorufin O-dealkylase (EROD) assay (Doostdar *et al.* 2000).

Our data suggested that the decreased expression of COMT and UGT1A1 might partially contribute to the stimulation on tumor growth in AD group. However, there was no association between the inhibition of dietary HES on tumor growth and COMT or UGT1A1 expression. UDP-Glucuronosyltransferase (UGT) is a

superfamily of enzymes that catalyze the glucuronidation of both endogenous toxins and xenobiotics. Induction of these enzymes is considered beneficial (Miners and Mackenzie 1991; Bock *et al.* 1999). Walle *et al* (2000) have reported an induction of UGT1A1 by the flavonoid chrysin in the human hepatic and intestinal cell lines HepG2 and Caco-2. Another four flavonoids acacetin, apigenin, luteolin, and diosmetin were also estimated to have the same effects as chrysin (Walle and Walle 2002). E2 has been reported to inhibit COMT expression and low expression of COMT was associated with an increased breast cancer risk (Wen *et al.* 2007; Wagner *et al.* 2008). These studies suggest the induction of COMT and UGT1A1 may correlate with flavonoids' putative protective function against breast carcinogenesis.

HES has been demonstrated to inhibit cell proliferation and G1-phase cell cycle arrest in MCF-7 cells. As HES has inhibitory effects on tumor growth in this present study, we further examined the expression of cell apoptotic and cell cycle-regulated gene to find whether the underlying mechanism is related to modulation of cell-cycle regulatory proteins (Jin et al. 2008). The eukaryotic cell cycle is controlled by different cyclin-dependent kinase (CDK)/cyclin complexes in response to exogenous growth stimulators, such as hormones, growth factors, and signals, at several key checkpoints (Okayama 1994; Grana and Reddy 1995). Cyclin D1 is an important regulatory protein involved in the G1 phase of cell cycle and its expression is always increased in many cancers (Hiyama et al. 1997). Estrogen has been reported to induce breast cancer cell proliferation via stimulating G(1)/S transition through increased expression of cyclin D1 and CDKs (Foster et al. 2001). $p21^{WAF1}$ and

$p57^{Kip2}$ are CDK inhibitory proteins which could bind and inactivate CDK/cyclin complexes (Matsuoka et al. 1998; Sherr and Roberts 1999). The blockage of G1/S transition could result in cell apoptosis (Mantena et al. 2006; Choi 2007).

Estrogen may also alter Bcl-2 family gene expression in favor of cell survival (Lee et al. 1998; Leung and Wang 1999). In the present study, the expression of cyclin D1 and CDK4 was increased while $P57^{Kip2}$ expression was inhibited in AD group. The co-treatment of HES could reverse the expression of these genes. This indicated that estrogen production was inhibited by dietary administration. The G1-phase cell cycle arrest (Kim 2005) induced by HES may lead to apoptosis in MCF-7 cells.

HES treatment at 1000ppm and 5000ppm significantly decreased the Bcl-xL expression induced by the AD, however, no significant differences were observed in Bcl-2, Bax and Bak expression. Our result suggested that decreased expression of Bcl-xL in the tumors could be a downstream effect of HES treatment. Bax expression was suppressed in the AD group, which implicated a role in stimulating tumor growth.

In conclusion, our results demonstrated that dietary treatments of HES inhibited aromatase enzyme activity and suppressed proliferation of MCF-7aro cells in vivo. It prevented the xenograft growth by downregulating the expression of cyclin D1, CDK4 and Bcl-xL and upregulating $p57^{Kip2}$ expression. Many of these genes were estrogen responsive. Since this model has been developed for studying breast

carcinogenesis in postmenopausal women, dietary HES could be a potential chemopreventive agent against postmenopausal breast cancer.

# CHAPTER 6
# EFFECTS OF PHYTOESTROGENS ON GROWTH OF MCF-7 CELLS IN CRUDE MATRIGEL AND ANDROGEN IN VIVO

## 6. 1 INTRODUCTION

Exposure to estrogen has been considered a risk factor for breast cancer (Ciocca and Fanelli 1997; Lee and Sheen 1997).This causal effect of estrogen exposure was supported in a transgenic model performed recently (Yoshidome *et al.* 2000). Estrogen can be hydroxylated into genotoxic intermediates (Zhou *et al.* 1996; Kao *et al.* 1998; Zhu and Conney 1998; Sebastian and Bulun 2001; Kijima *et al.* 2006). In spite of its mutagenic potential, estrogen has long been regarded as a cancer promoter (Li and Li 1987; Liehr 2000). It induces proliferation of breast cancer cells and alters Bcl-2 family protein expressions in favor of anti-apoptosis (Leung and Wang 1999). Although alternate pathways have been suggested, the receptor-mediated nuclear event is still the core of the hormone's physiological action (Ciocca and Fanelli 1997). Therefore, the antiestrogen tamoxifen is usually administered as an adjuvant therapy for receptor-positive breast cancers (Margreiter 1980). On the other hand, a contemporary treatment targeting the estrogen synthesis enzyme aromatase has been developed.

Many Phase-I and-II enzymes are responsible for the metabolism of estrogen. Aromatase (CYP19) is the critical enzyme synthesizing estrogens by converting C19 androgens to aromatic C18 estrogenic steroids (Zhou *et al.* 1996; Kao *et al.* 1998;

Sebastian and Bulun 2001; Kijima *et al.* 2006). Increased CYP19 expression has been demonstrated in breast cancer tissue, and estrogen concentration in the tissue is many times higher than the circulation (Miller and Dixon 2001). Santner *et al.* (1993) and Yue *et al.* (1998) have illustrated that locally produced estrogen encourages tumor growth. Lee *et al.* (2003) and Hirose *et al.* (2004) have shown that polymorphisms in the *CYP19* gene are associated with increased risk of breast cancer. CYP17 converts pregnenolone and progesterone to precursors of androgen and estrogen (Chen *et al.* 2008). The genotype polymorphism of CYP17, which induced high activity, is associated with an elevated risk for breast cancer in Taiwanese women (Huang *et al.* 1999). However, the association was not observed in another study (Chen *et al.* 2008). CYP1A1 is a phase I enzyme that hydroxylates estrogen at the position of C-2, and its expression is inducible by PAH through the aryl hydrocarbon receptor (AhR) activation pathway (Spink *et al.* 1998; Nebert *et al.* 2004). Polymorphisms with augmented CYP1A1 inducibility in African-Americans (Taioli *et al.* 1995) and Chinese (Huang *et al.* 1999) are associated with increased breast cancer risk. However, this association fails to hold in Indian women (Singh *et al.* 2007) or Korean women (Shin *et al.* 2007). CYP1B1 hydroxylates estrogen at the C-4 position to form 4-hydroxyestrogen. The hydroxylated metabolite of estrogen retains all the estrogenic properties and can be more damaging with regard to its genotoxicity than the parent compound (Liehr 2000; Roos and Bolt 2005). Constitutive CYP1B1 is mainly expressed in peripheral tissues, such as the breast (Huang *et al.* 1996). Polymorphisms of *CYP1B1* are associated with increased risk of

breast cancer (De Vivo *et al.* 2002; Zimarina *et al.* 2004). In a separate study, exposure to xenobiotics may also increase the risk of breast cancer in women with polymorphically active CYP1B1 (Saintot *et al.* 2004). These observations are consistent with the properties of CYP1B1. UGT1A1 catalyzes the formation of estrogen-glucuronide. A low expression of UGT1A1 may increase the circulating estrogen. Africa Americans (Guillemette *et al.* 2000; Shatalova *et al.* 2006) who have the polymorphic *UGT1A1* associated with reduced expression have an increased risk of breast cancer. (Yueh *et al.* 2001) have suggested that early down regulation of the UGTs in premalignant and malignant tumor tissues may be a prerequisite of cellular carcinogenesis. We have demonstrated that an elevated level of UGT1A1 in MCF-7 cells was associated with decreased cell proliferation (Leung *et al.* 2007). The catechol estrogens generated by CYP1 enzymes can be genotoxic. COMT inactivates these catechol estrogens by methylation (Hamajima *et al.* 2001). Subsequently, the methylated catechol estrogens will be glucuronidated and eliminated by UGT. A recent study on the *COMT* SNPs has revealed that polymorphisms with higher expression of COMT were associated with reduced breast cancer risk in premenopausal women (Ji *et al.* 2008). However, negative results have also been reported as reviewed by Gallicchio *et al.* (2006).

Several phytoestrogens are available in the market as dietary supplements for alleviating postmenopausal symptoms. The flavanone naringenin (NAR) is the precursor compound of genistein in some plants. It is found abundantly in citrus fruits, especially in grapefruit (78 mg/100 g). The estimated peak plasma

concentrations in individuals after consuming orange juice or grapefruit juice (8 ml/kg) are 0.6 and 6.0 μM, respectively (Erlund *et al.* 2001). Genistein (GEN) and biochanin A can be isolated from soyabean and red clover, respectively. They are two of the most commonly use ingredients in phytoestrogen supplement. However, most biochanin A will be converted into genistein after absorption. The dietary relevance of the genistein dosages in human subjects has been reasonably established over many years of research. The major metabolite in women is in the glucuronide form, and the aglycone genistein only constitutes about 25% of total genistein present in plasma (Zhang *et al.* 2003). In a high-soya consuming country such as Japan, the average plasma concentration of total genistein is about 0.5μM in women (Pumford *et al.* 2002). Supplementation may bring about 1μM in the plasma of human subjects (Izumi *et al.* 2000). Isoliquiritigenin (ILN) is a flavonoid isolated from licorice, which is the sweet-tasting rhizome of the bean plant *Glycyrrhiza glabra*.

MCF-7 cells are commonly used in breast cancer research. However, they express a limited amount of aromatase as compare to most estrogen-responsive tumors (Miller and Dixon 2001). Mai et al. (Mai *et al.* 2007) developed a MCF-7 transplant model with 17β-estradiol supplementation for investigating breast carcinogenesis; it is still a common model nowadays. In this model, ovariectomy is performed to resemble the hormonal status in post-menopausal women. 17β-estradiol pellet is implanted as compared to hormone replacement therapy. Because of the controvercy of androgen administration in postmenopausal women, androstenedione was replaced estrogen in the present study. In addition, there is an increased interest of phytoestrogen

consumption in post- menopausal women. We use this modified animal model to examine the interaction between androgen and phytoestrogens in breast carcinogenesis in this group of population. We hypothesized that androgen increased estrogen supply and phytoestrogen would further increase the potency of cell proliferation. The experimental design was carried out as below:

**Animal model.** This study was designed to determine whether the flavonoid can act as a therapeutic or chemopreventive agent. Twenty-four female nude mice aged around 6 weeks were acquired from Animal Facility of Chinese University of Hong Kong. The mice were ovariectomized and allowed 3 weeks to recover. The mice were randomly divided into 4 groups: C, NAR, ILN, and GEN. Mice received subcutaneous injection with vehicle in C group and other groups received androstenedione injection (0.1 mg/mouse) every other day throughout the experiment. The injection of androstenedione is to supply the substrate for estrogen synthesis. The advantage for this protocol was that the estrogen synthesis step was included in the ultimate evaluation. Phytoestrogens (6 per group) were administered in a semipurified AIN-93G diet and the feeding regimen (0, 5000ppm NAR, 5000ppm ILN, 5000ppm GEN) started right after the xenograft transplantation. All the groups were transplanted with MCF-7 cells. Before transplantation, the MCF-7 cells were maintained in culture incubator. After growing to 80% confluency, the cells were trypsinized, suspended in matrigel matrix (10 mg/ml) and 0.1 ml of cells ($3 \times 10^7$ cells/ml) were injected into the two flanks of the back of each animal. The crude matrigel was prepared as follows: 10g Engelbreth–Holm–Swarm (EHS) mouse

sarcoma was homogenized in high salt buffer with protease inhibitor (0.05M Tris-Hcl, PH7.4, 3.4M Nacl, 4μM EDTA, 2μM NEM). Then it was centrifuged at 7000g for 15 min, the supernant was discarded and the pellet was suspended in 30ml salt buffer. This step was repeated twice. Finally the pellet was suspended in 10 ml urea buffer (0.15 M Nacl, 0.05M Tris-HCL, 4M urea, pH 7.4) and stirred overnight at 4ºC. The mixture was then centrifuged at 14000g for 20 min and the supernant was saved. The pellet was homogenized in 5 ml urea buffer and centrifuged at 14000g for 20min. The supernatant was dialysed against 10 volume of Tris-buffered saline with 5 ml chloroform per litre and it was repeated without chloroform for 4 times every 2 hours. Finally it was dialysed against 10 volume of DMEM overnight. Dialysis bag was rinsed with ethanol and matrigel was diluted to 10 mg/ml concentration.The matrigel was stored at -20ºC for further use. The mice body weight and tumor size of the mice were monitored weekly and food intake were measured through the study. Tumor volumes were measured by an electronic caliper and estimated according to the formula: $\pi$ /(6×length× width × height), where length, width, and height were the three orthogonal diameters of the tumors. At the end of the study, the mice were sacrificed by cervical dislocation 93 days post-transplantation. Tumors, blood, liver and uterus samples were collected for assays. The procedure was approved by Animal Experimentation Ethics Committee, Chinese University of Hong Kong.

## 6.2 RESULTS

### 6.2.1 ERE-driven Luciferase Activities Induced by NAR, ILN and GEN in HepG2 cells Expressing ERα

A reporter gene assay was set up for testing the ER transactivation of NAR, ILN and GEN. HepG2 cells, previously documented with no ER expression, were employed for this assay. After transfected with ERα plasmids and ERE-driven reporter plasmids, cells were treated with the three phytoestrogens. Result as shown in **Fig.6.1** indicated that all three phytoestrogens were able to initiate ER transactivation with genistein (GEN) to be the strongest.

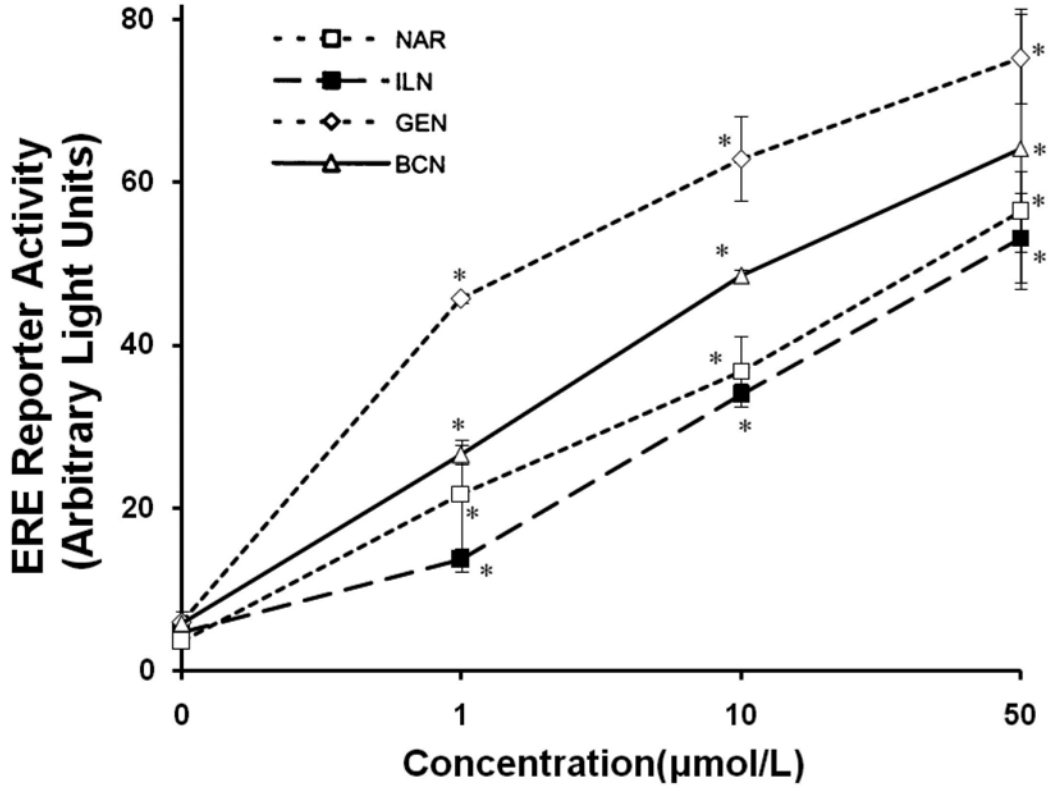
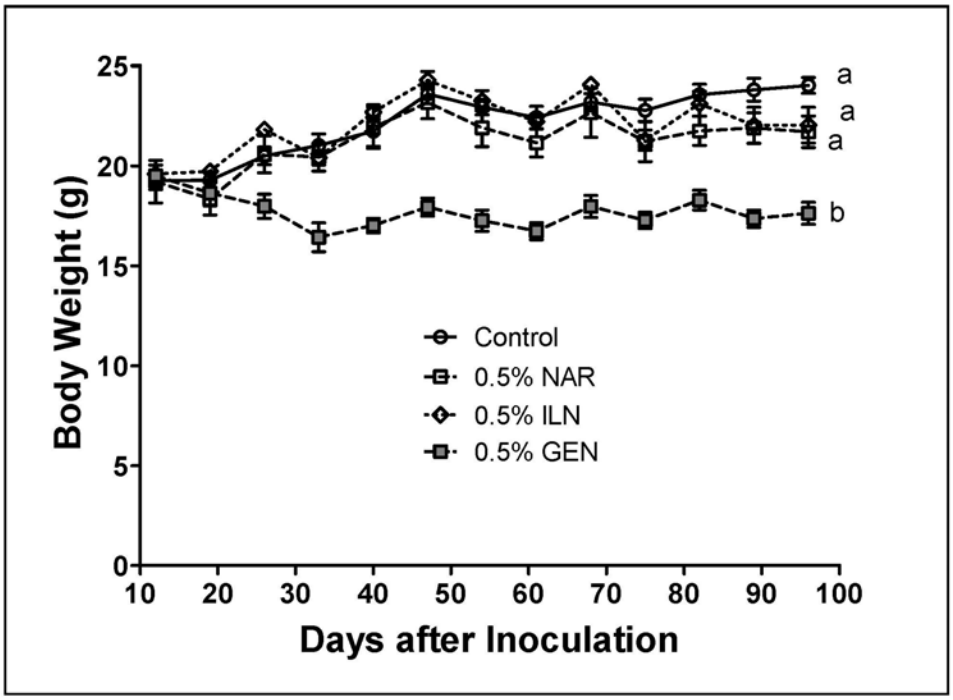



**Fig.6.1** HepG2 cells were plated in 24-well dishes. After 24 hrs, the cells were transiently transfected with 0.25μg ER-expression plasmid, 0.25μg ERE-driven reporter plasmid and 2.0ng of renilla luciferase control vector pRL. After 1 day, the medium was removed and the cells were treated with different phytochemicals for 24 hrs. The cells were lysed and the activities of the luciferases were determined using Dual-Luciferase Assay Kit (Promega). One-way ANOVA followed by Bonferroni's Multiple Comparison Test if significant differences ($p < 0.05$) were observed. Values are means ± SEM, n=3. *Means were significantly ($p < 0.05$) different from that of the control.

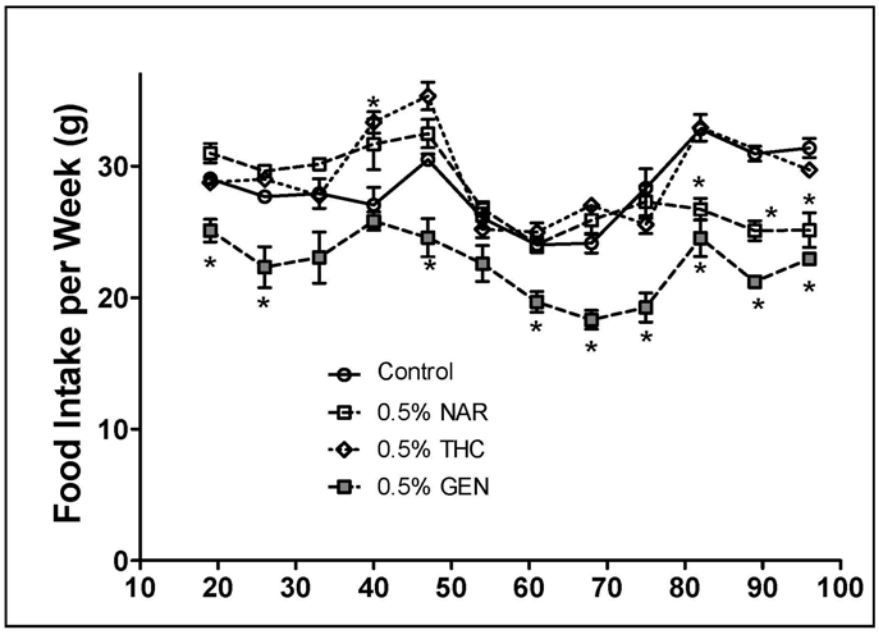
### 6.2.2 Effect of NAR, ILN, and GEN on mice body and liver weight

The mice body weights were measured weekly since checked out from the animal facility. GEN mice also had lower body weights starting from day 27 after cell innoculation (**Fig.6.2.a**), which is consistent to the results from feed consumption. GEN mice consumed 15-30% less feed than the control throughout the experiment (**Fig.6.2.b**). There was no significant difference in body weight and feed consumption with NAR or ILN treatment. Similar liver weights among various phytoestrogen treatments (**Fig.6.2.c**) indicate that the dosages were tolerable to the nude mice.

a



b




c

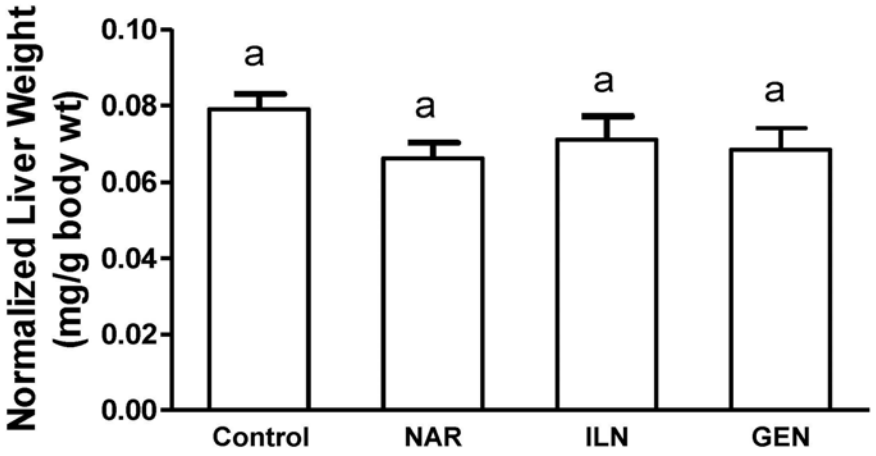
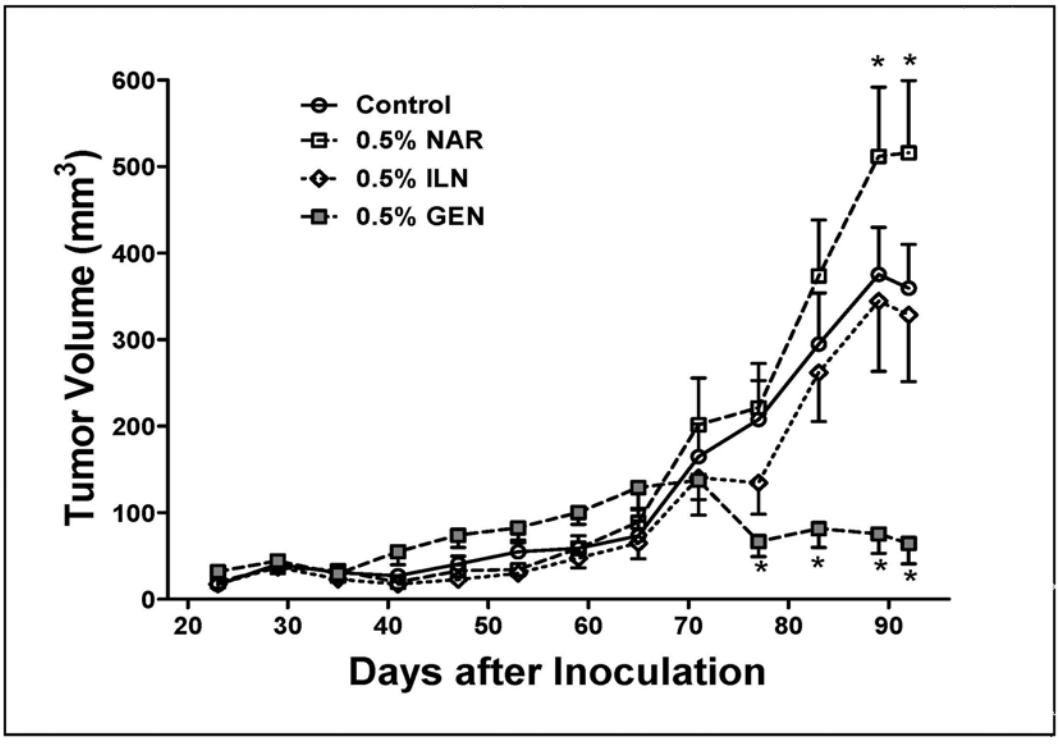



**Fig.6.2 Effect of NAR, ILN, and GEN on nude mice's body, food intake and liver weight.** Mice were inoculated with MCF-7 cells and treated with 5000ppm NAR, ILN, and GEN. Their body weights and food intake were monitored from the first week after inoculation. The liver weights were measured at the end of experiment. Values are means ± SEM, n=6. The data collected was analyzed by One-way ANOVA, followed by Tukey's Multiple Comparison test.

### 6.2.3 Effects of NAR, ILN and GEN on MCF-7 tumor growth in ovariectomized nude mice

Androstenedione (0.1 mg/mouse) was injected *s.c.* every other day to supply the substrate for estrogen synthesis in all the group. 5000ppm NAR significantly increased the tumor size by about 1.5 at day 89 and 92 while ILN treatments had no significant effects on tumor size **(Fig.6.3.a)**. The tumor cell transplant in nude mice fed 5000ppm GEN, on the other hand, displayed a reduction in tumor size at day 77 post-inoculation **(Fig.6.3.a)**. The tumor weights in Control group were heavier than those in GEN group ($P<0.05$), which was consistent with the results from the tumor growth **(Fig.6.3b)**. No difference was revealed in tumor weights among the mice with Control, NAR and ILN treatment.

a



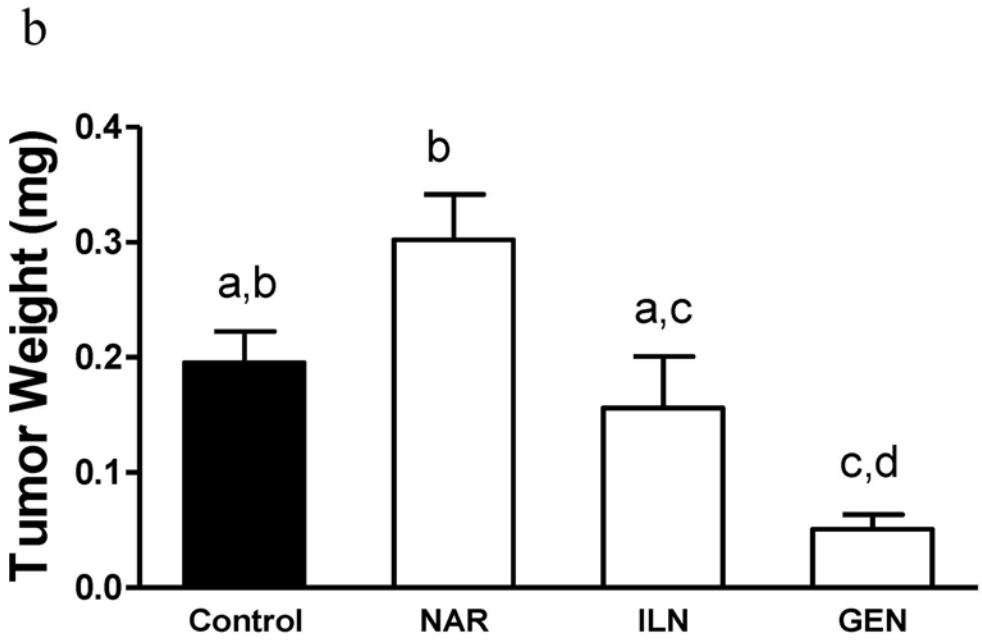



**Fig.6.3 Effects of NAR, ILN and GEN on MCF-7 tumor growth in ovariectomized nude mice.** Mice were inoculated with MCF-7 cells at 2 sites per mouse after ovariectomy. They were fed with diet with 5000ppm NAR, ILN, and GEN and androstenedione was injected *s.c.* every other day on the next day after inoculation. Tumor volumes were estimated two times a week from Day 23 after inoculation (Fig.7.3a). The tumor weights were measured at the day of sacrifice (Fig.7.3b). Values are means ± SEM, n=11 to 12. The data was analyzed by One-way ANOVA, followed by Tukey's Multiple Comparison test. Means labeled with different letters are significantly different.

### 6.2.4 Plasma E2 levels

Serum collected from group GEN and NGN displayed a significant increase of estradiol concentration compared with group Control **(Fig.6.4)**. There was no statistically difference observed in E2 levels in NGN or ILN group compared with Control group.

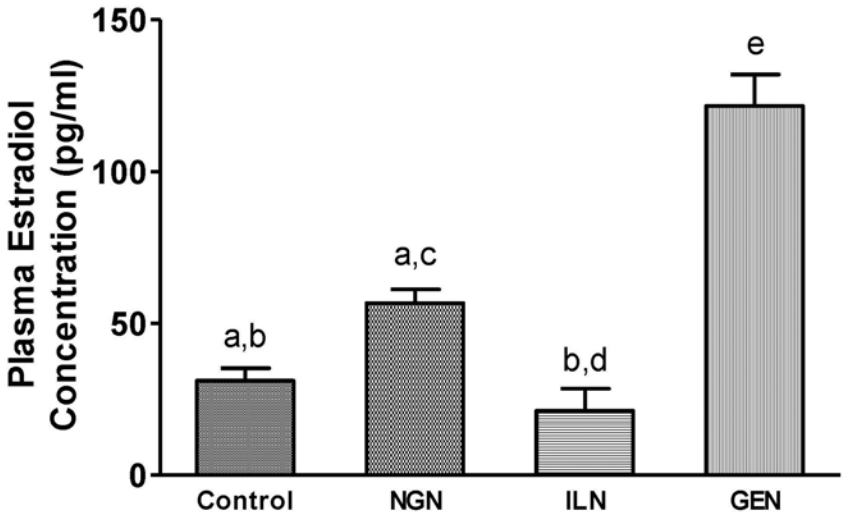



**Fig.6.4.Serum estradiol concentration in ovariectomized mice**. Blood was drawn from the animals at sacrifice. Serum estradiol concentration was quantified by ELISA. Values are means ± SEM, n=6.The data was analyzed by One-way ANOVA, followed by Tukey's Multiple Comparison test. Means labeled with different letter are significantly different.

### 6.2.5 Uterine Wet Weight

Dietary GEN treatment (5000ppm) significantly increased uterine wet weight in ovariectomized MCF-7 implanted mice with AD injection. This trend of uterine weight was consistent to the corresponding serum estradiol concentration between Control and GEN group. There was no statistical difference observed among Control, NAR and ILN group **(Fig.6.5)**.This result suggested that GEN may have uterotrophic or estrogenic action, which was mediated by its interaction with uterine ER, thereby increase uterus weight in GEN group. Previous studies have demonstrated that genistein a well-documented ER agonist.

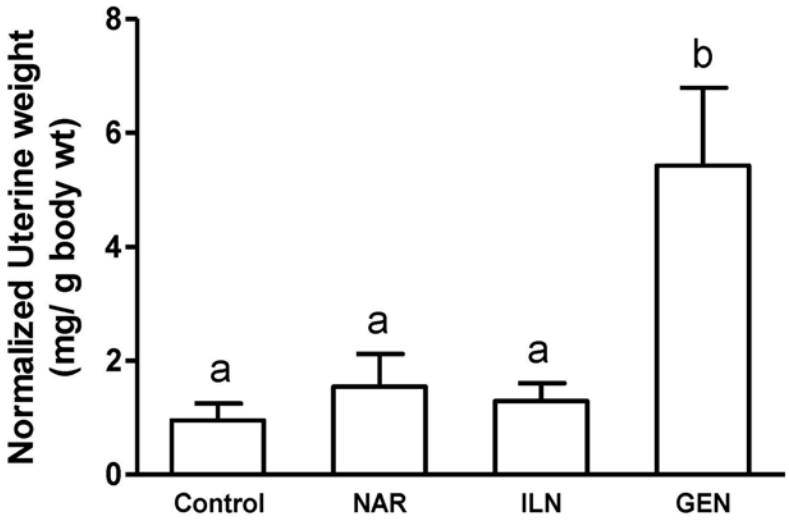


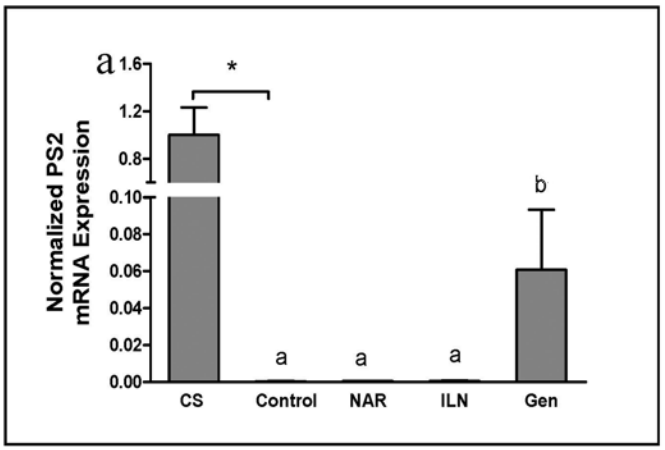

**Fig.6.5. Uterine weight in ovariectomized nude mice.** Uteri of the experimental animals were dissected and weighed at sacrifice. Values are means ± SEM, n=6. The data was analyzed by One-way ANOVA, followed by Tukey's Multiple Comparison test. Means labeled with different letter are significantly different.

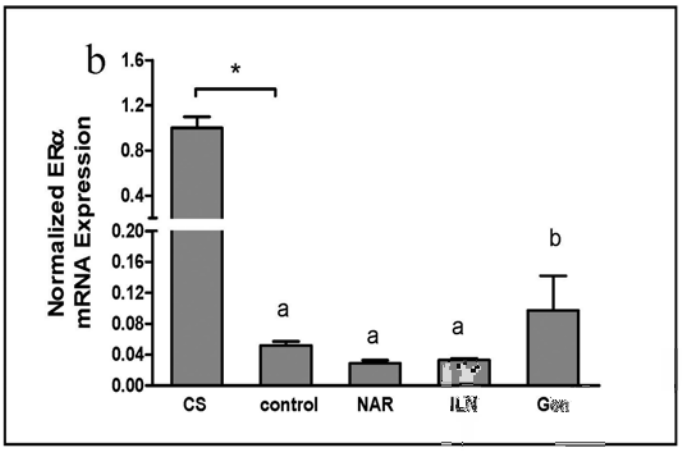
### 6.2.6 Determining effects of NAR, ILN and GEN on E2 responsive and metabolic-related gene expression

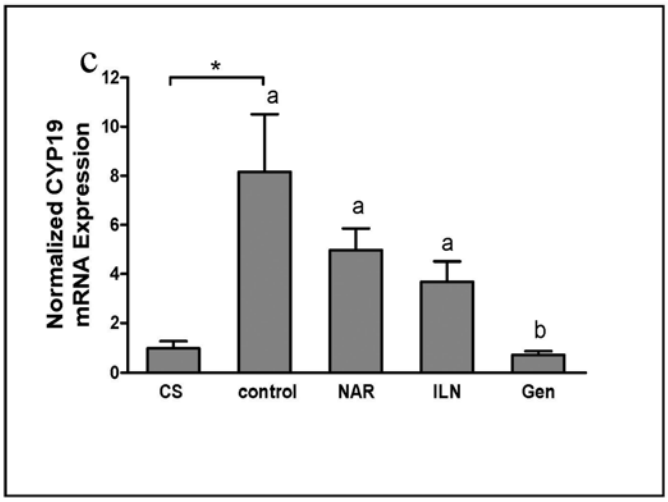
Total mRNA was extracted from tumors and evaluated for the E2 responsive and metabolic gene expression. The following E-responsive genes pS2 and E2 metabolic gene, ERα, CYP19, CYP17, CYP1A1, CYP1B1, COMT, UGT1A1 and Bcl-2, were evaluated by using real-time PCR (**Fig.6.6**).

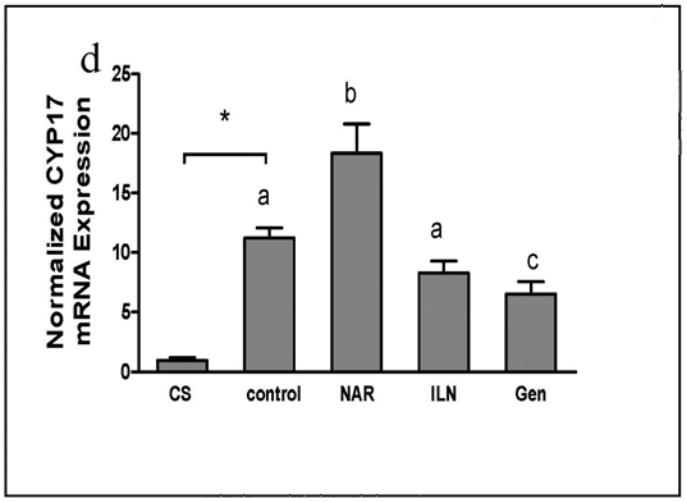
**pS2.** pS2 expression was significantly increased in GEN group **(Fig.6.6a)** ($P < 0.05$). There was no statistical difference observed among Control, NAR and ILN group.

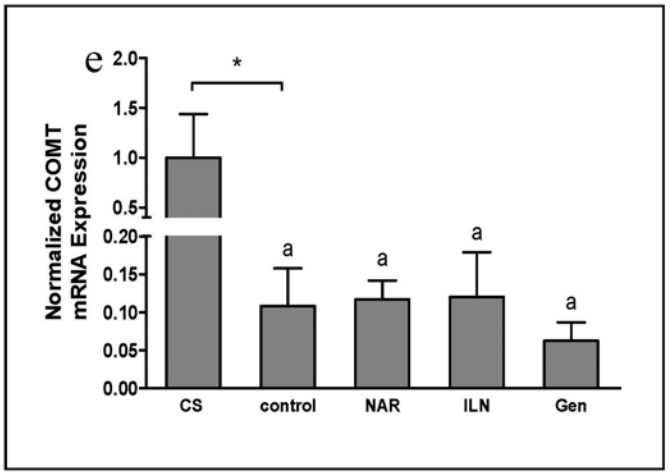
**ERα, CYP19, CYP17, CYP1A1, CYP1B1, COMT, UGT1A1 and Bcl-2.** A significant increase in ERα expression was observed in GEN group compared with Control group **(Fig.6.6b)** ($p<0.05$). A decrease in the CYP19, CYP17, CYP1B1 and Bcl-2 was observed in GEN group compared with Control group **(Fig.6.6 c, d, g, h)**. NAR treatment displayed an increase in CYP17 expression **(Fig.6.6d)** and a decrease in UGT1A1 expression **(Fig.6.6f)**. There was no difference in CYP1A1 expression among four groups(data not shown).There was also no statistical difference observed in ERα, CYP19, CYP1B1, COMT and BCl-2 expression among Control, NAR and ILN groups.

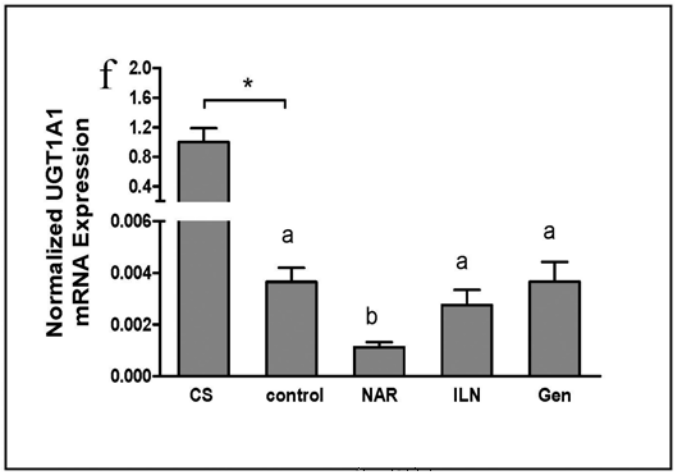

a
Normalized PS2 mRNA Expression
1.6
1.2
0.8
0.10
0.08
0.06
0.04
0.02
0.00
*
a
a
a
b
CS
Control
NAR
ILN
Gen


b
Normalized ERα mRNA Expression
1.6
1.2
0.8
0.4
0.20
0.16
0.12
0.08
0.04
0.00
*
a
a
a
b
CS
control
NAR
ILN
Gen


c
Normalized CYP19 mRNA Expression
12
10
8
6
4
2
0
*
a
a
a
b
CS
control
NAR
ILN
Gen


d
Normalized CYP17 mRNA Expression
25
20
15
10
5
0
*
a
b
a
c
CS
control
NAR
ILN
Gen


e
Normalized COMT mRNA Expression
2.0
1.5
1.0
0.5
0.20
0.15
0.10
0.05
0.00
*
a
a
a
a
CS
control
NAR
ILN
Gen


f
Normalized UGT1A1 mRNA Expression
2.0
1.6
1.2
0.8
0.4
0.006
0.004
0.002
0.000
*
a
b
a
a
CS
control
NAR
ILN
Gen

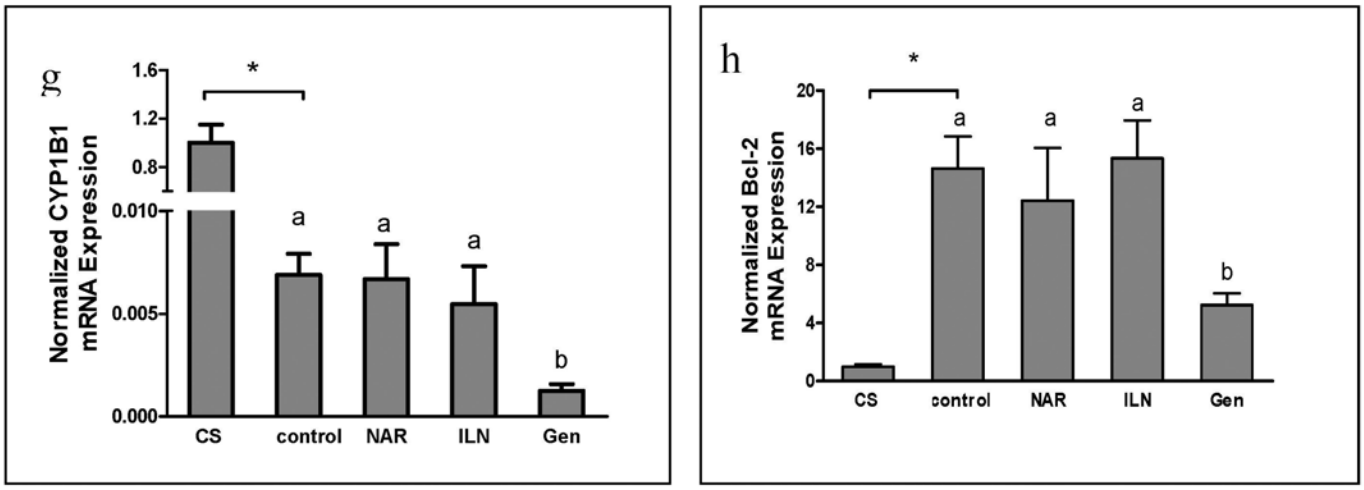



**Fig.6.6. Relative pS2, ERα, CYP19, CYP17, CYP1A1, CYP1B1, COMT, UGT1A1 and Bcl-2 mRNA levels in tumors.** Total mRNA was extracted from tumors and messenger RNA expression of these genes was quantified by real-time PCR. Values are means ± SEM, n=5 to 6.The data was analyzed by One-way ANOVA, followed by Tukey's Multiple Comparison test. Means labeled with different letter are significantly different.

## 6.3 Discussion

In this study, we demonstrated that three phytoestrogens tested had differential growth effects on MCF-7 tumors grown on crude matrigel. GEN treatment appeared to reduce the tumor size; whereas ILN and NAR fed mice either had no effect or increased the xenograft growth.

Previous study (Maggiolini *et al.* 2002) has demonstrated that ILN is an agonist to ER isoforms. It stimulates MCF-7 cell proliferation at low concentrations and is cytotoxic at high concentrations. Our lab has previously demonstrated that ILN treatment was able to suppress the proliferation of the aromatase-overexpressing MCF-7 cells *in vivo* (Ye *et al.* 2008). In contrast, ILN administration did not suppress the growth of the wild-type MCF-7 cells with androstenedione supplementation in the current study. It appeared that the estrogen synthesis pathway did not significantly contribute to the growth of the wild-type MCF-7 tumor.

Similar to ILN, GEN is also demonstrated to be ER agonist and displays biphasic effect on MCF-7 cell proliferation (Leung *et al.* 2005). Previous studies have shown that GEN treatment could increase MCF-7 xenograft transplant in ovariectomized athymic mice (Hsieh *et al.* 1998), and reduces the chemotherapeutic potential of the ER antagonist tamoxifen and the aromatase inhibitor letrozole (Allred *et al.* 2001; Ju *et al.* 2006). However, null (Gallo *et al.* 2006) and suppressive (Shao *et al.* 1998) effects of the isoflavone on MCF-7 tumor growth have been observed in two other labs. Our findings support results of the latter studies. However, body weight reduction due to reduced food intake could be the major

contributing factor.

NAR is weakly estrogenic as demonstrate in a assay with ER expressed in yeast (Zierau *et al.* 2002). It inhibits MCF-7 cell growth *in vitro* (So *et al.* 1997; Harmon and Patel 2004), and the suppression of glucose uptake could be the underlying mechanism. NAR can also potentially inhibit breast cancer cell growth by suppressing the drug efflux (Chung *et al.* 2005), inhibiting estrogen synthesis (van Meeuwen *et al.* 2007), and activating peroxisome proliferator-activated receptor gamma (Liu *et al.* 2008). In spite of all these potential protective factors, the present study indicated that naringenin facilitated rather than suppressed MCF-7 xenograft growth. Since the plasma estradiol was not increased, naringenin likely potentiated the tumor growth in an estrogen-independent pathway.

Many flavonoids are weak agonists on estrogen receptor. However, NAR and ILN had null effect on the uterine weight as shown in this study. Disregard findings in the reporter gene assays, the result indicated that these two phytoestrogens were not estrogenic *in vivo*. On the other hand, increased uterine weights were only seen in mice under GEN treatment.

As an increase of circulating estrogen level was observed in GEN-treated mice, we evaluated expressions of genes responsible for metabolizing estrogen in tumors. Gene expressions of CYP19, CYP17 were reduced in the tumors of GEN as compared to Control. The localized estrogen synthesis in the tumor might be compromised with the decrease. On the other hand, NAR-treated mice displayed an increased CYP17 expression and a decrease in UGT1A1 expression. The amount of

estrogen at the tumor might have sustained at a high level with the differential expressions. Previous study has shown that localized estrogen synthesis is detrimental to breast cancer growth (Santner *et al.* 1993; Yue *et al.* 1998). These gene expression profiles might be partially, if not entirely, responsible for the differential tumor sizes among the three phytoestrogen treatments.

Several factors could separate the results of the present study from those of the other comparable studies. The transplant model for MCF-7 cells was modified by androstenedione administration to stimulate estrogen synthesis in this study. The hormone might exercise some physiological functions other than acting as the substrate of estrogen synthesis alone. It may stimulate some signaling kinases through androgen receptor independent of estrogen pathways. Moreover, we employed crude matrigel freshly isolated from Engelbreth–Holm–Swarm (EHS) mouse sarcoma. The growth factor-rich matrigel could support the growth of MCF-7 tumor without estrogen supplementation.

In summary, the present study demonstrated that phytoestrogens could differentially affect ER-positive breast cancer cell growth transplanted in ovariectomized nude mice. These data could be significant in establishing the safety aspects of dietary phytoestrogen supplement.

# CHAPTER 7
# THE SOY ISOFLAVONE GENISTEIN INDUCES ESTROGEN SYNTHESIS IN AN EXTRAGONAL PATHWAY

## 7.1 INTRODUCTION

Since GEN significantly increased the serum estrogen concentration in ovariectomized athymic mice, we further investigated its effect on estrogen synthesis through an extragonadal pathway. We employed the hepatocytes HepG2 to test effects of GEN on the aromatase expression and underlying mechanism as well. Epidemiological studies have shown that the use of exogenous estrogen (Pike and Spicer 1993) or augmented endogenous estrogen concentration (Toniolo *et al.* 1995) is associated with increased breast cancer risk. In both cell and animal models a causal relationship between estrogen exposure and breast cancer has also been established (Colditz 1999). The notion that estrogen promotes breast cancer is reinforced in a transgenic mouse model that develops spontaneous mammary tumors. Treatment of estrogen accelerates the development of neoplastic lesions and carcinomas in these mice (Yoshidome *et al.* 2000). Estrogen-induced cell proliferation has been a major focus in breast cancer research. The pertained mechanisms lie in the regulation of cell cycle (Dickson *et al.* 1987; Tsai and O'Malley 1994), Bcl-2 family protein expression (Leung and Wang 1999), and the interaction with plasma membrane receptors (Watson *et al*. 1999).

On the other hand, estrogen has its physiological functions. For instance,

estrogen replacement therapy has long been used for controlling postmenopausal symptoms, including lowering blood cholesterol. (Lamon-Fava *et al.* 1999) have demonstrated that estrogen increases promoter activities of *ApoA-1* in HepG2 cells. An estrogen receptor (ER)-independent pathway has also been described (Zhang *et al.* 2001) for equine estrogen in the up-regulation of *ApoA-1* promoter activity.

Genistein is a major soy isoflavone that has been the focus of many studies regarding its health benefits. The isoflavone shares some common structure with the hormone estrogen. Despite the similarity, the relative binding affinity of genistein to ER-α is only 0.05-1% of the binding affinity of 17β-estradiol (Shutt and Cox 1972). In contrast, its binding affinity to ER-β is greater than that of estrogen. It is suggested that genistein may act as a selective estrogen receptor modulator (SERM) (Kuiper *et al.* 1998). Epidemiologic studies have associated the consumption of isoflavonoids with a lower incidence of CVD (Adlercreutz *et al.* 1998). In normal postmenopausal women, consuming whole soy foods with 60 mg isoflavones per day may help alleviating several key clinical risk factors for CVD (Scheiber *et al.* 2001). Apolipoproteins and receptor proteins, which may directly affect the blood cholesterol concentration, are synthesized in the liver. As the expressions of these proteins can be regulated by estrogen, we would like to examine the effect of genistein on hepatic CYP19 in the present study.

## 7.2 RESULTS

### 7.2.1 Effect of genistein on aromatase activity in HepG2 cells

Genistein could induce aromatase activity in HepG2 cells. 1 and 10 μM genistein significantly increased the activity by 1.2 to 1.75 fold, respectively (**Fig.7.1**).

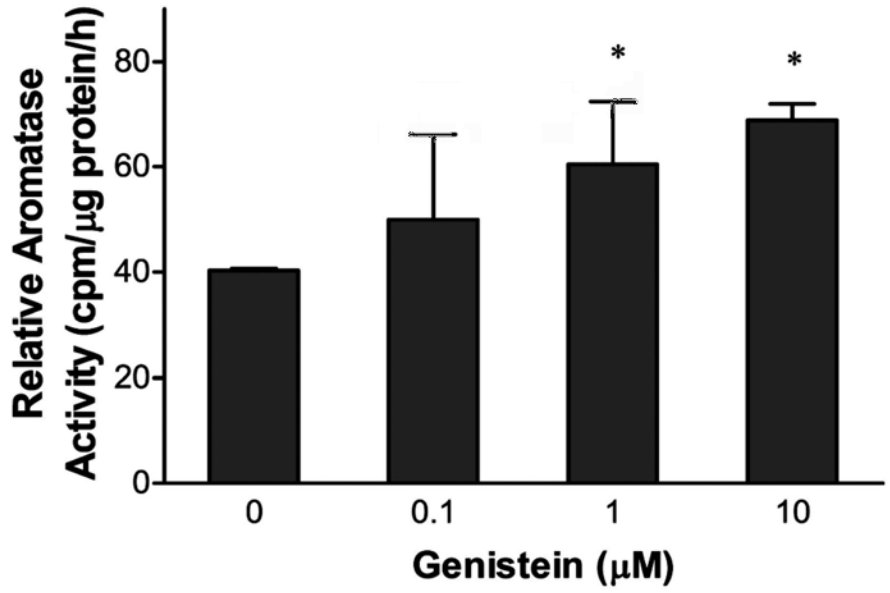



**Fig.7.1** Effect of genistein on aromatase activity in HepG2 cells. HepG2 cells were cultured and treated with genistein for 24 h before tritiated androstenedione was added. At the enzyme level, genistein could significantly increase the activity. (*) signifies a significant ($p < 0.05$) increase in activity when compared with the control. One-way ANOVA followed by Bonferroni's Multiple Comparison Test if significant differences ($p < 0.05$) were observed. Values are means± SEM, n = 3. Data represents one of two independent experiments with comparable results.

### 7.2.2 CYP19 promoter utilization in HepG2 cells treated with genistein

Prior to quantifying the mRNA expression of CYP19 induced by genistein, we measured the alternate spliced mRNA species in HepG2 cells. Promoter usage had not been investigated in liver cells before. Exon II was the dominating mRNA species followed by Exon I.3 in HepG2 cells with or without genistein treatment (**Fig.7.2**). Another liver cell line WRL68 also displayed similar promoter usage (data not shown). This indicated that Exons I.3 and II were controlling the transcriptional activity of *CYP19* in this cell type.

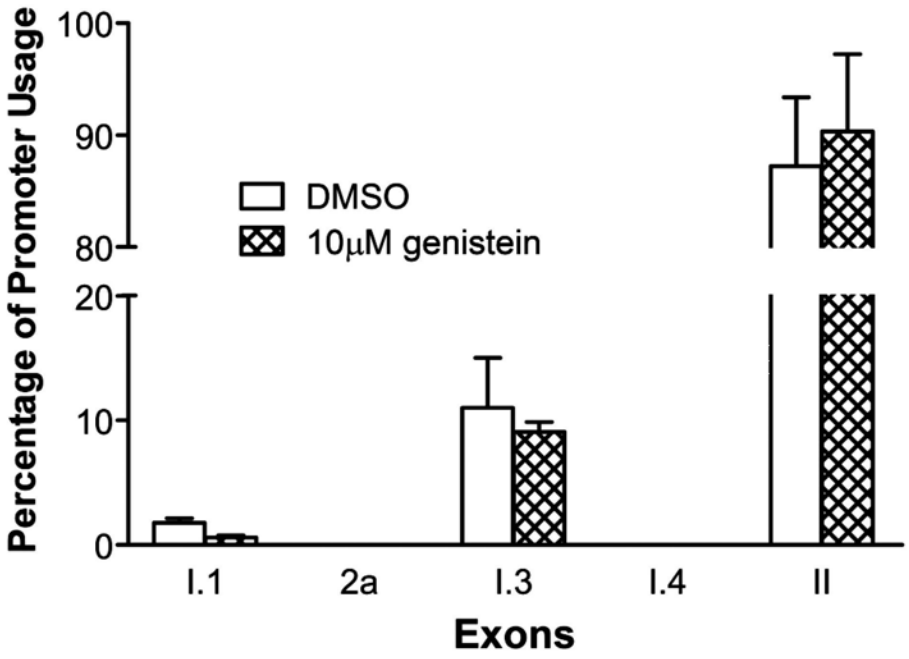
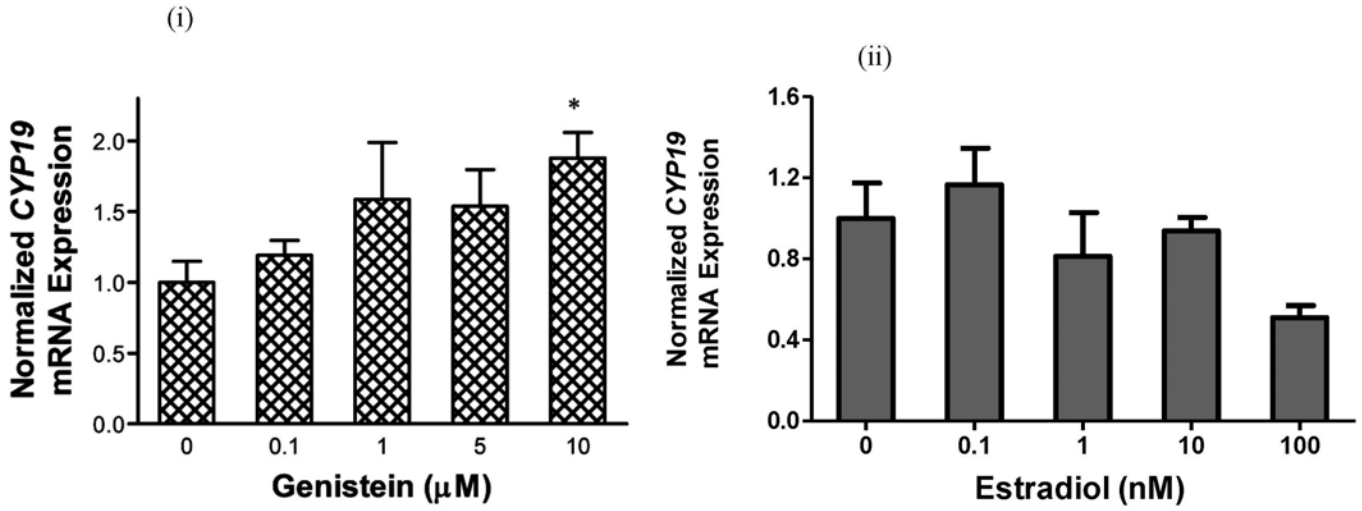



**Fig.7.2.** Relative promoter usage in HepG2 cells. HepG2 cells were cultured and treated with genistein for 24 h. Messenger RNA was extracted and various promoter specific species were determined by real-time PCR. The relative expression for each species was normalized with GAPDH. Values are means± SEM, n = 3. Data represents one of two independent experiments with comparable results.

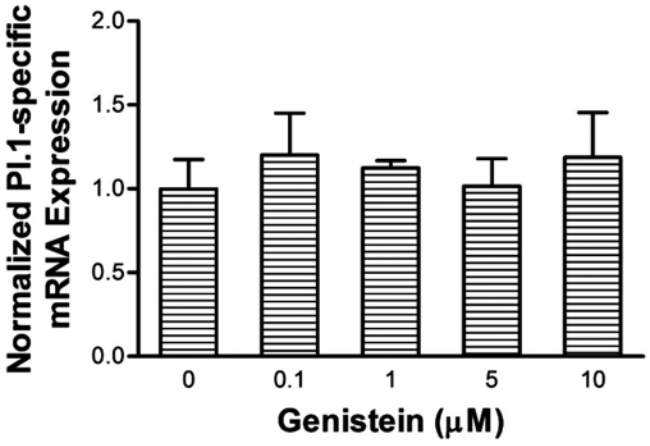
### 7.2.3 Genistein induced aromatase mRNA expression

Quantitative RT-PCR indicated that them RNA abundance of aromatase coding region was increased by genistein. Cultures treated with 10μM genistein revealed a significant elevation in the expression (**Fig.7.3A(i)**). In contrast, estradiol ranging from 0.1 to 100nM could not significantly increase the expression (**Fig.7.3A(ii)**). This indicated the induction was initiated through a pathway independent of estrogen receptor (ER). Subsequently, the alternate spliced mRNA species were measured. Significant increases were observed in exons I.3 (**Fig.7.3C**) and II (**Fig.7.3D**) specific species treated by 10μM genistein, whereas exon I.1 specific mRNA was not upregulated (**Fig.7.3B**).

A


(i)
Normalized CYP19 mRNA Expression
2.0
1.5
1.0
0.5
0.0
*
0
0.1
1
5
10
Genistein (μM)
(ii)
Normalized CYP19 mRNA Expression
1.6
1.2
0.8
0.4
0.0
0
0.1
1
10
100
Estradiol (nM)

B


Normalized PI.1-specific mRNA Expression
2.0
1.5
1.0
0.5
0.0
0
0.1
1
5
10
Genistein (μM)

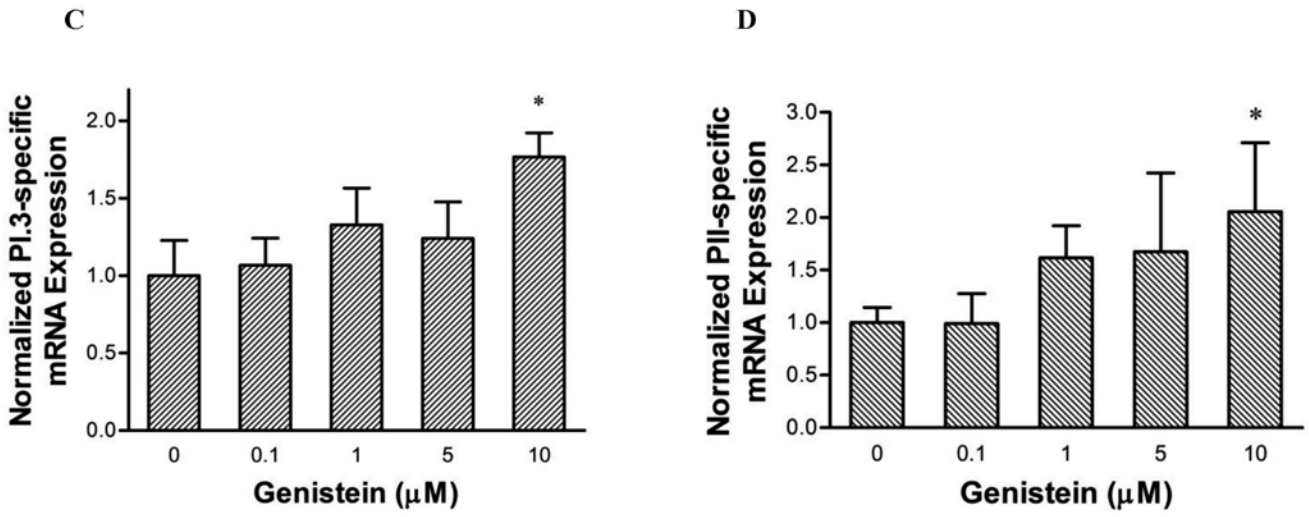
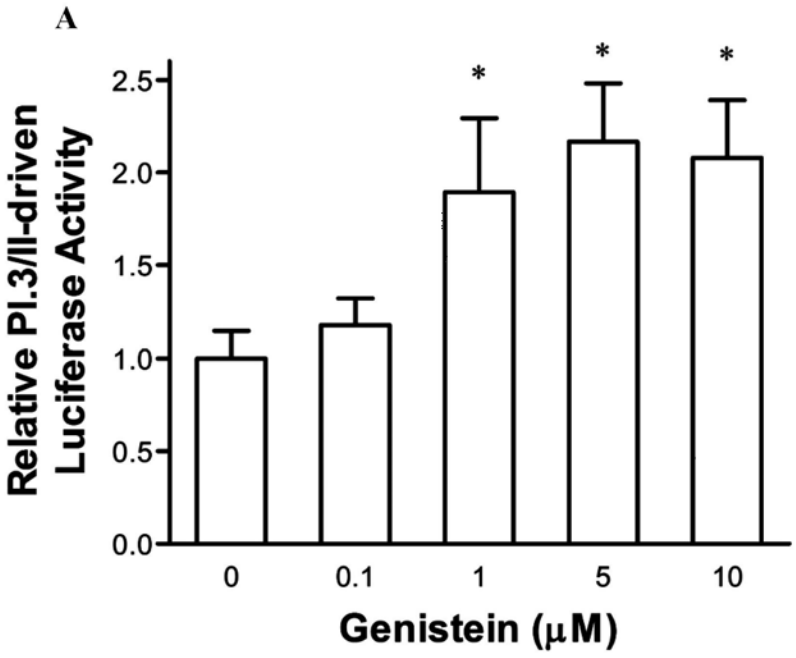



**Fig.7.3.** Genistein-induced aromatase exon I.3 and II mRNA expression in HepG2 cells. HepG2 cells were seeded in six-well culture plates and were treated with genistein. Messenger RNA was extracted and quantitated by real-time PCR. (A) Genistein increased CYP19 expression. (*) represents a significant ($p < 0.05$) increase in expression when compared with the control cultures. Genistein upregulated the alternate spliced mRNA driven by PI.3 (B) and PII (C) but not PI.1 (D). One-way ANOVA followed by Bonferroni's Multiple Comparison Test if significant differences ($p < 0.05$) were observed. Values are mean± SEM, $n = 3$. The experiment was repeated once with comparable results.

### 7.2.4 Reporter gene assay on promoters I.3/II in HepG2 cells

In order to verify the induced exon expression, promoter I.3/II-driven reporter gene assay was carried out. Genistein could significantly increase the promoter transactivity (**Fig.7.4A**). Further investigation on the truncated promoter indicated that a differentiated response was observed in the segment -56/-100 (**Fig.7.4B**).



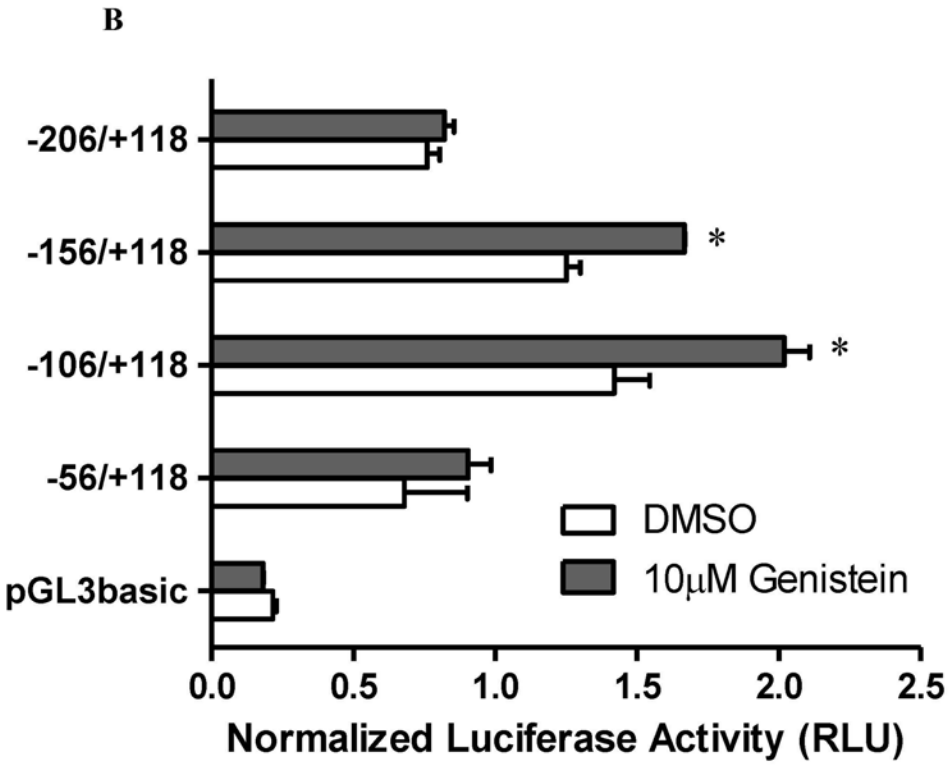
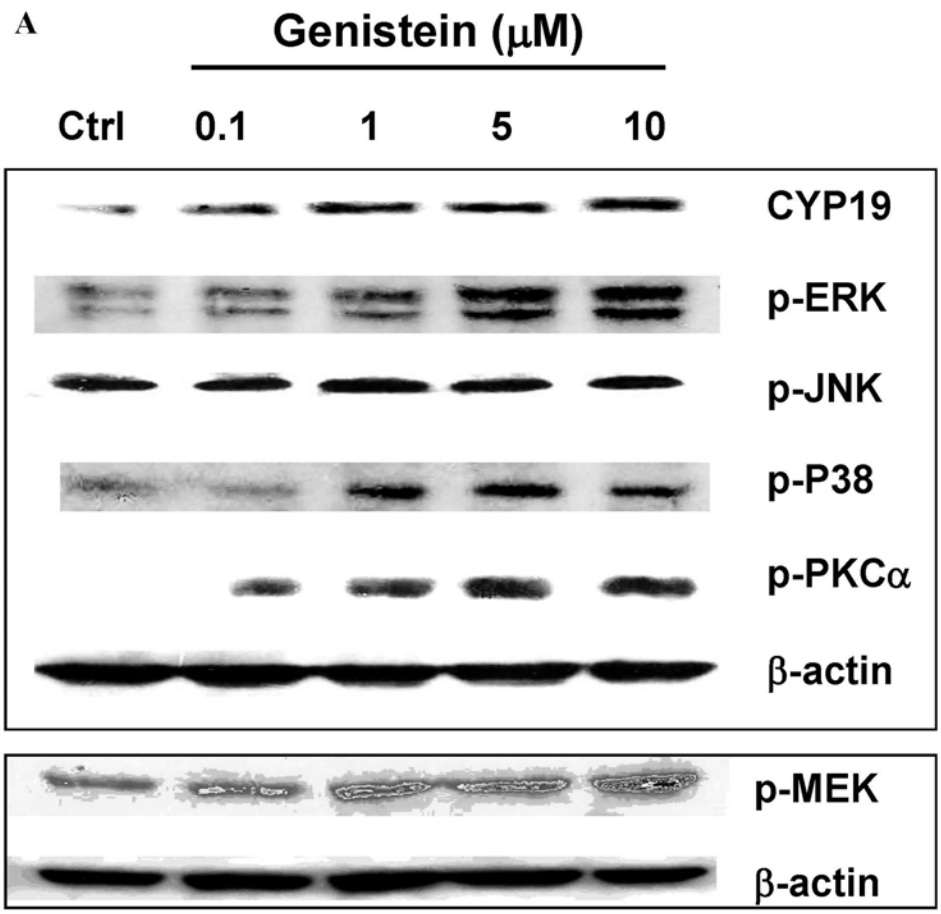



**Fig.7.4** Reporter gene assay of promoters I.3/II in HepG2 cells. HepG2 cells were seeded in 24-well culture dishes and transfected with PI.3/II-driven reporter plasmid, and renilla luciferase plasmid. After transfection, the cultureswere treated with genistein for another day. The cells were lysed and assayed for firefly and Renilla luciferase activities (**A**). Mean luciferase activity labeled with (*) is significantly ($p < 0.05$) increased when compared with the control cultures. Subsequently, a series of truncation reporter plasmids of CYP19 PI.3/II were constructed and transfected into HepG2 cells. The cells were then treated with 10μM genistein and assayed for luciferase activities (**B**). Means labeled (*) represents a significant ($p < 0.05$) increase in activity when compared with the corresponding control. One-way ANOVA followed by Bonferroni's Multiple Comparison Test if significant differences ($p < 0.05$) were observed. Values are mean ± SEM, $n = 3$. The experiment was repeated once with comparable results.

### 7.2.5 Activation of protein kinases in HepG2 cells treated with genistein

As various experiments demonstrated that CYP19 was upregulated, we also looked into the possible regulatory mechanisms. Because ERK was previously implicated in the regulation of CYP19 in breast cells, we looked into several signaling kinases by immunoblotting in the present study. Genistein activated some protein kinases concurrently with the elevation of the CYP19 protein (**Fig.7.5A**). Among the kinases tested, the phosphorylated forms of PKCα, P38, MEK and ERK-1/2 were induced. Optical density of the images as shown in **Fig.7.5B** illustrated that the amount of these activated kinases were significantly increased.


A
Genistein (μM)
Ctrl
0.1
1
5
10
CYP19
p-ERK
p-JNK
p-P38
p-PKCα
β-actin
p-MEK
β-actin

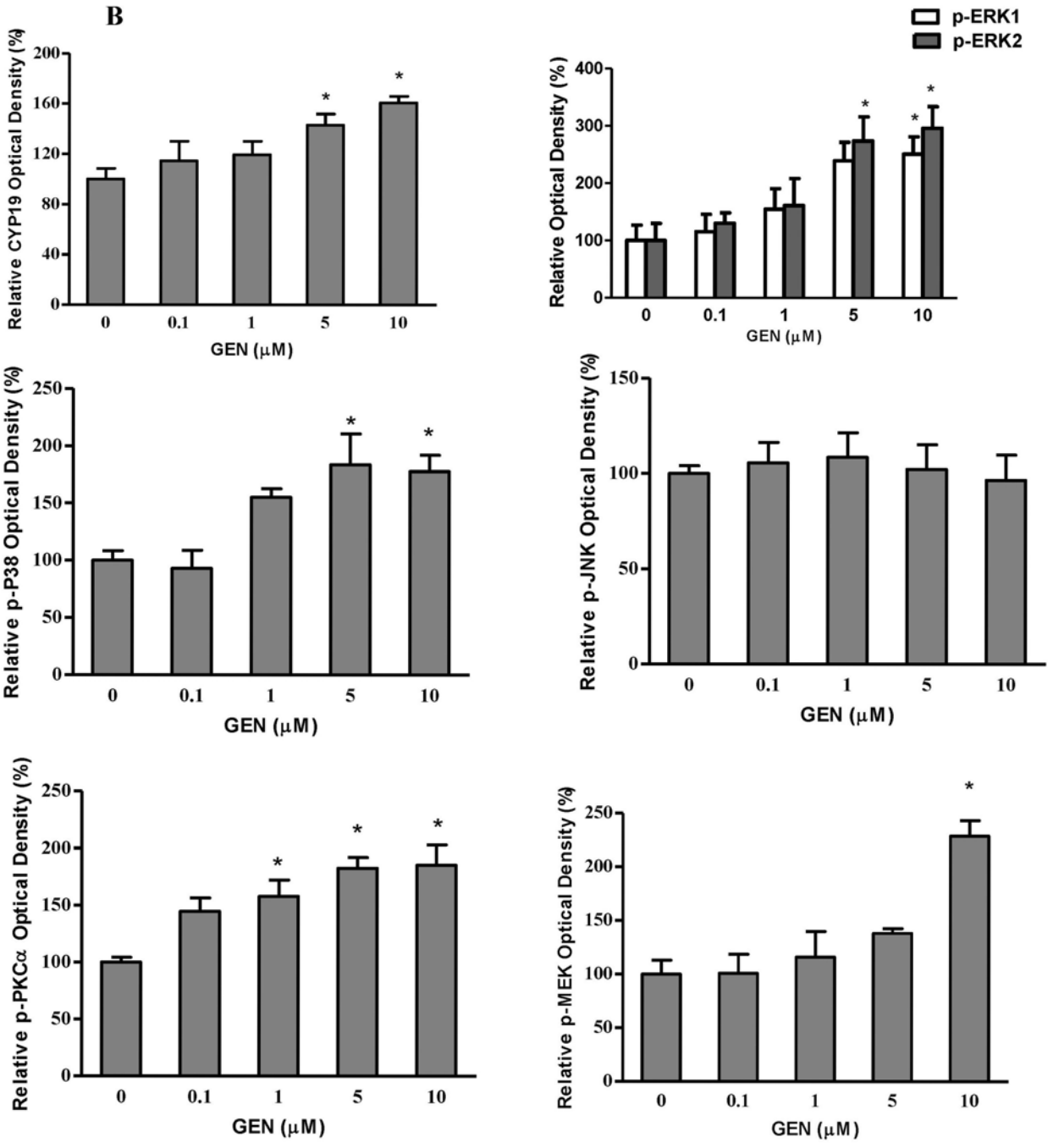
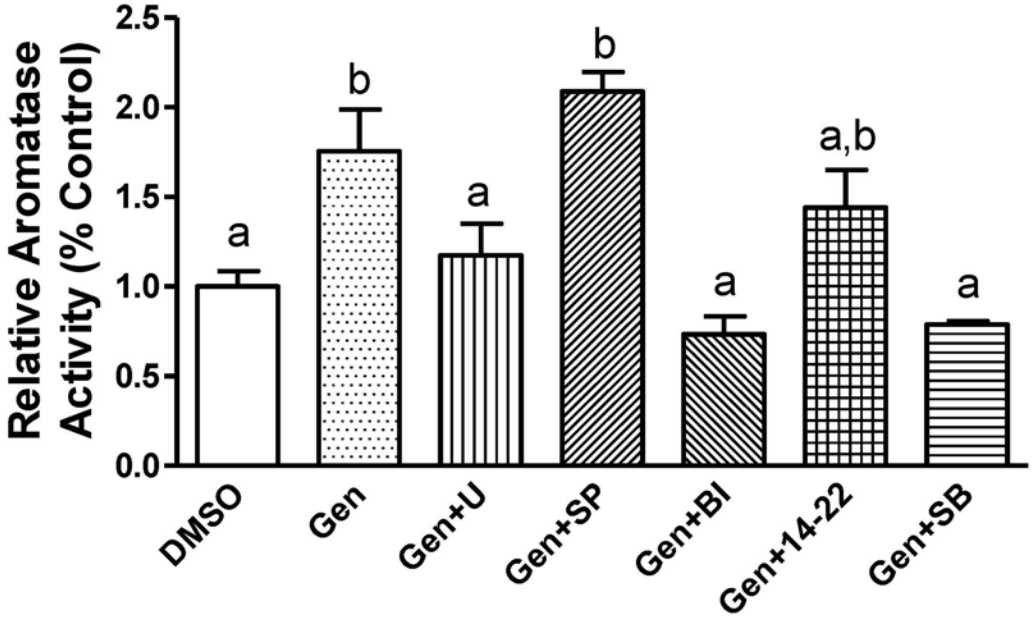



**Fig.7.5.** Immunoblot of signaling proteins in HepG2 cells treated with genistein. HepG2 cells were seeded in six-well culture dishes and treated with genistein for 24 h. Protein expression of aromatase, p-ERK-1/2, p-JNK, p-P38, p-PKCα and p-MEK in cell lysates was determined by Western blot analysis. The images in (**A**) are representations of three independent experiments, and figures in (**B**) are the optical density readings of the proteins. Values are mean ± SEM, n = 3. Mean with (*) is significantly ($p < 0.05$) different from control.

### 7.2.6 Inhibiting PKCα, P38 and ERK suppressed CYP19 expression in HepG2 cells

Since the activation of several protein kinases was observed, we further verified their significance by inhibiting these kinases. In cultures pre-treated with the inhibitor of ERK, PKCα, or P38, the genistein-upregulated CYP19 expression (**Fig.7.6B**) and activity (**Fig.7.6A**) were reduced. These results complemented the data presented in **Fig.7.5**, and verified that genistein might potentiate aromatase activity through activation of protein kinases.

**A**



**B**

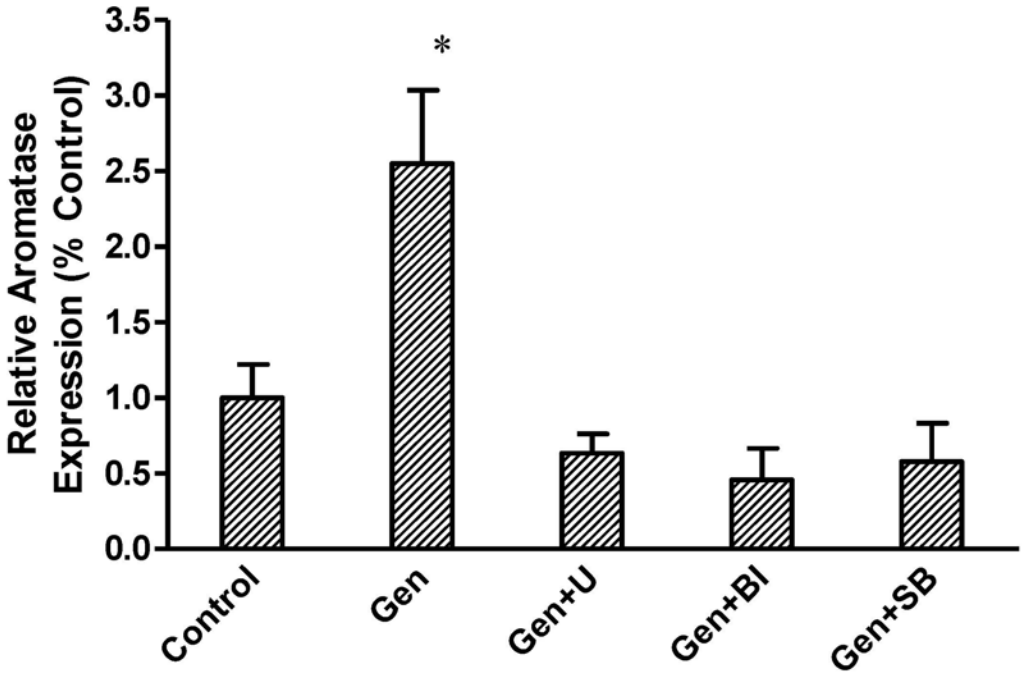



**Fig.7.6.** Verification of PKCα, ERK and MEK involvement in genistein-induced CYP19 expression. HepG2 cells were seeded in six-well culture dishes and pre-treated with the ERK inhibitor U0126 (U), the PKC inhibitor bisindolyl-maleimide I (BI), the JNK inhibitor SP600125 (Chen *et al.*), the PKA inhibitor myristoylated 14–22 amide (14–22), or the P38 inhibitor SB203580 (SB), followed by genistein (GEN) treatment. Messenger expression and activity of aromatase were determined (**A**). In (**B**), messenger RNA was isolated and quantified for CYP19 expression after 24 h of incubation. Means labeled (*) represents a significant ($p < 0.05$) increase in expression or activity when compared with the control. One-way ANOVA followed by Bonferroni's Multiple Comparison Test if significant differences ($p < 0.05$) were observed. Values are mean ± SEM, n = 3. Data represents one of two independent experiments with comparable results.

### 7.2.7 DNA binding activity and protein expression of CREB

The truncation reporter plasmids in **Fig.7.4** implied that (-105/-56) of *CYP19* Promoter II might be responsible for the induced transcriptional activity, and EMSA assay was performed to investigate the regulation. In consideration of the potential transcriptional factors downstream to ERK, PKCα, or P38, an AP-1 or CRE binding site was located within this region by using the promoter scanning software, MATCH™ (Biological Databases GmbH, Wolfenbuttel, Germany).

Nuclear extract isolated from genistein-induced cells displayed a band shift with a synthetic oligonucleotide of *CYP19* Promoter II (-91/-56). Supershift phenomenon could only observe by using antibody raised against CREB-1, but not Jun antibody (**Fig.7.7**). This result showed that CREB instead of AP-1 was activated. In coupling with the supershift band, intensity of the major bands was diminished.

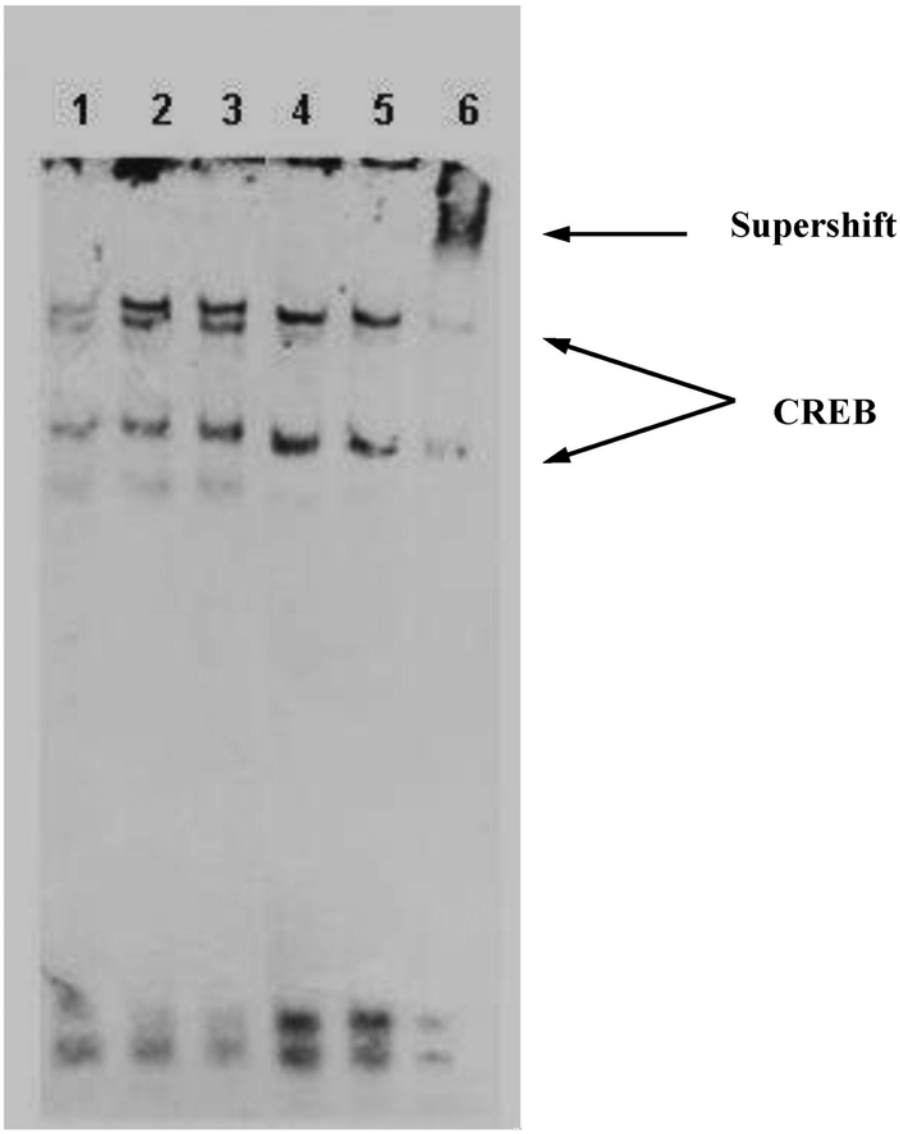



**Fig.7.7.** CREB was involved in genistein-induced CYP19 transcription. After identified the genistein-activated segment of the promoter, binding activity in this segment was investigated. Nuclear extracts were prepared from HepG2 cells treated with genistein and EMSA was performed. Lanes 1–5 are samples treated with genistein at 0, 1, 5 and 10μM; lane 6 is a sample treated with 10μM genistein incubating with CREB antibody. Data represents one of two independent experiments with comparable results.

### 7.2.8 Western blot on nuclear CREB in genistein-treated cells

Western analysis indicated that CREB protein was induced in nuclear extracts isolated from genistein-treated cells in a dose-response manner (**Fig.7.8**). The lighter band below could be the inactive or unphosphorylated protein.

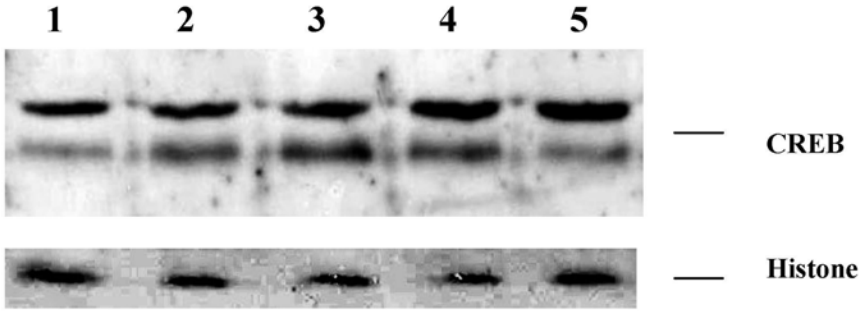



**Fig.7.8.** Western analysis on nuclear CREB under genistein treatment. HepG2 cells were treated with genistein for 24 h. Nuclear extracts were isolated and immunoblot on CREB was performed. Lanes 1–5 are genistein treatment at 0, 0.1, 1, 5 and 10μM. Histone was used as the normalization protein. Data represents one of two independent experiments with comparable results.

## 7.3 DISCUSSION

In this study, we illustrated that genistein was able to induce aromatase activity in HepG2 cells. At the transcriptional level, the phytoestrogen increased the aromatase mRNA abundance in these hepatocytes. We further demonstrated that the induced expression could be mediated by the protein kinases PKCα, ERK and P38. In contrast to its protein tyrosine kinase inhibitory properties, genistein in the tested range could induce PKCα phosphorylation, and might subsequently activate P38, MEK, and ERK.

There are ten distinct tissue-specific promoters in *CYP19*. Promoters I.1, I.2, and IIa are mainly used by placental cells. Adipocytes can use promoter I.3 or I.4, while breast cancer cells are regulated by promoters I.3 and II (Shozu *et al.* 1998; Sebastian and Bulun 2001; Sebastian *et al.* 2002). At the transcriptional level, many factors have been described for the regulation of aromatase. Simpson *et al.* (1997) have reviewed that cyclic AMP, phorbol esters, dexamethasone, $PGE_2$, transforming growth factor-β, and γ-interferon increase the transcriptional activity, whereas cyclo-oxygenase inhibitors suppress the mRNA expression (Kinoshita and Chen 2003; Diaz-Cruz *et al.* 2005) have previously reported that ERK inhibitor may reduce CYP19 transcription in breast cells. In the present study, exons I.3 and II were identified to be the transcriptional control of *CYP19* in the hepatic cells HepG2. Activation of ERK and P38 with the subsequent increased CREB activity appeared to be the underlying mechanisms.

As reviewed by Mayr and Montminy (2001), CREB is a phosphorylation-dependent transcriptional factor with multiple isoforms and interacts

with CRE motifs in the promoter region of a gene. Studies have suggested that CREB monomers could bind to the palindromic CRE in different time points and dimerize on the site subsequently. Several kinases, such as PKA, PKC, P38 have been implicated in the phosphorylation process. In the present study genistein could stimulate the DNA binding of CREB and CYP19 transcription through activating PKC and P38. The downstream signaling protein ERK appeared to be a part of the transcriptional regulation, since the ERK inhibitor U0126 could offset some of the induced aromatase activity and expression. CREB is not a substrate of ERK, but the phosphorylation of the transcriptional factor can take place by way of the protein kinase MSK-1. MSK-1, which is downstream to ERK, can activate CREB. Besides, genistein could also activate ERK independent of PKCα in HepG2 cells (Lamon-Fava and Micherone 2004).

Many phytochemicals have been reported to be aromatase inhibitors at the enzyme or transcriptional level, or both. Extract of red wine inhibits aromatase activity (Eng *et al.* 2001), and reduces mammary hyperplasia in transgenic mice over-expressing CYP19. The active ingredients in the extract can be procyanidin B dimmers (Eng *et al.* 2003) and resveratrol (Wang *et al.* 2006). Chalcones, which are a subclass of flavonoid, display inhibitory actions on aromatase in placental microsomes with $IC_{50}$ values greater than or equal to 34.6 M (Le Bail *et al.* 2001). Kao *et al.* (1998) have shown that the flavonone naringinin is a stronger inhibitor than the chalcones. In contrast to a previous report on isoflavones in MCF-7 cells (Wang *et al.* 2008),

genistein in the current study induced rather than suppressed aromatase activity. Differences in cell type could separate the results.

Differential effect of genistein on aromatase has been reported in gonadal cells. In human endometrial stromal cells (Edmunds *et al.* 2005) and immature rat ovarian follicles (Myllymaki *et al.* 2005), genistein administration can increase aromatase activity. In contrast, Rice *et al.* (2006) report that the activity is reduced when human granulose-luteal cells are exposed to genistein. As the aromatase expression is cell-specific, these inconsistent results can be explained by the promoter-specific regulation of CYP19. Besides, conflicting effects of genistein on aromatase have also been described in MCF-7 cells in two separate studies (Brueggemeier *et al.* 2001; Brooks and Thompson 2005). Variations in drug dosage and time of treatment could contribute to the outcome.

Genistein can favorably alter the cholesterol metabolism in HepG2 cells (Lamon-Fava *et al.* 1999; Borradaile *et al.* 2002). It also increases -oxidation by upregulating carnitine palmitoyl transferase 1A (Shin *et al.* 2006) and suppresses fatty acid synthase in the same cell system (Shin *et al.* 2007). Some of these physiological effects can be induced by estrogen. Liver and peripheral cells are not considered to be a significant source of this hormone; however, the synthesis in extragonadal cells may become important when the ovaries have ceased to produce the hormone.

In summary, the present study demonstrated that genistein induced the transcription of *CYP19*. The endogenous production of estrogen could be important in regulating gene expressions in liver cells.

# CHAPTER 8
# SUMMARY

The female hormone estrogen may initiate various physiological functions, and excessive exposure to this hormone is a documented risk factor for breast carcinogenesis. Xenoestrogens and phytoestrogens are two major classes of endocrine disruptors. In the present study, effect of these two classes of compound on estrogen metabolism was investigated.

TCDD is a prototype compound of dioxin-like xenoestrogens. We studied the effect of TCDD on aromatase expression in the brain and adipose tissue in ovariectomized Sprague Dawley rats at different exposure timepoints. Real-time PCR and western blot analysis indicated that pre-ovariectomy administration of TCDD could significantly reduce aromatase expression in the brain but increase the expression in the adipose tissue. Since aromatase is responsible for estrogen synthesis, our results suggested that the timing of exposure to the toxicant could determine the estrogenicity of TCDD.

Phytoestrogens are another class of endocrine disruptors. They may function as antiestrogens or weak estrogens by competing with estrogen for estrogen receptor (ER) binding. In another study, we evaluated the effects of phytoestrogens ILN, HES, GEN and NAR on MCF-7 tumor growth in ovariectomized nude mice. ILN and HES were shown to be effective aromatase inhibitors in MCF-7aro cells. Both ILN and HES significantly deterred the xenograft growth of MCF-7aro cells transplanted in

ovariectomized nude mice. We further investigated the underlying mechanisms. The suppression mechanism of ILN on CYP19 transcription in MCF-7 cells is shown in **Fig.8.1**. We identified that a reduced activation of C/EBP might be responsible for the downregulation of CYP19. The expression of E2 responsive and E2 metabolism genes in tumors indicated that G1-phase arrest in cell cycle, apoptosis and reduced E2 production might contribute to HES's suppressive effect on the tumor growth. Tumor growth in this mouse model is dependent on the estrogen synthesized locally, which mimicks the situation in postmenopausal breast cancer patients.

We also investigated the effects of GEN, NAR, and ILN on xenograft growth using a separate model. MCF-7 cells were implanted into the ovariectomized athymic mice with constant androstenedione injection. Modifying from the established estrogen-supplementation model, the process of estrogen synthesis was encompassed in this model. As the expression of aromatase in MCF-7 cells was significantly lower than that in the MCF-7aro cells, the xenograft growth was less dependent on the estrogen synthesis pathway. We found that NAR at 5000 ppm increased the tumor size, and ILN did not suppress the growth of the MCF-7 xenograft as observed in the MCF-7aro xenograft model. This indicated that the tumor growth in this model was independent on estrogen synthesis. GEN at 5000 ppm significantly deterred the xenograft growth accompanying with 20% reduction in body weight. This study demonstrated that GEN could be protective against ER-positive breast tumor growth, but the desirable effect could be the result of caloric restriction.

Since GEN significantly increased the serum estrogen concentration in ovariectomized athymic mice, we further investigated its effect on estrogen synthesis through an extragonadal pathway. We employed the hepatocytes HepG2 for this study, and an induced extragonadal pathway of estrogen synthesis was identified as shown in **Fig.8.2**. The phytoestrogen could activate CREBs to bind to PII to initiate the transcription of CYP19.

In summary, the present results demonstrated that the action of xeno- and phyto-estrogens on estrogen metabolism wass specific on the target tissue and the timing of exposure. ILN and HES could inhibit the aromatase activity and deterred the xenograft growth of MCF-7aro cells transplanted in ovariectomized nude mice. GEN also inhibited MCF-7 xenograft growth in an estrogen independent manner, whereas NAR stimulated the growth. Phytoestrogens or flavonoids are major components of Chinese Medicine, fruits and vegetables, and herbs. Therefore, their therapeutic applications on hormone-related diseases should be evaluated carefully.

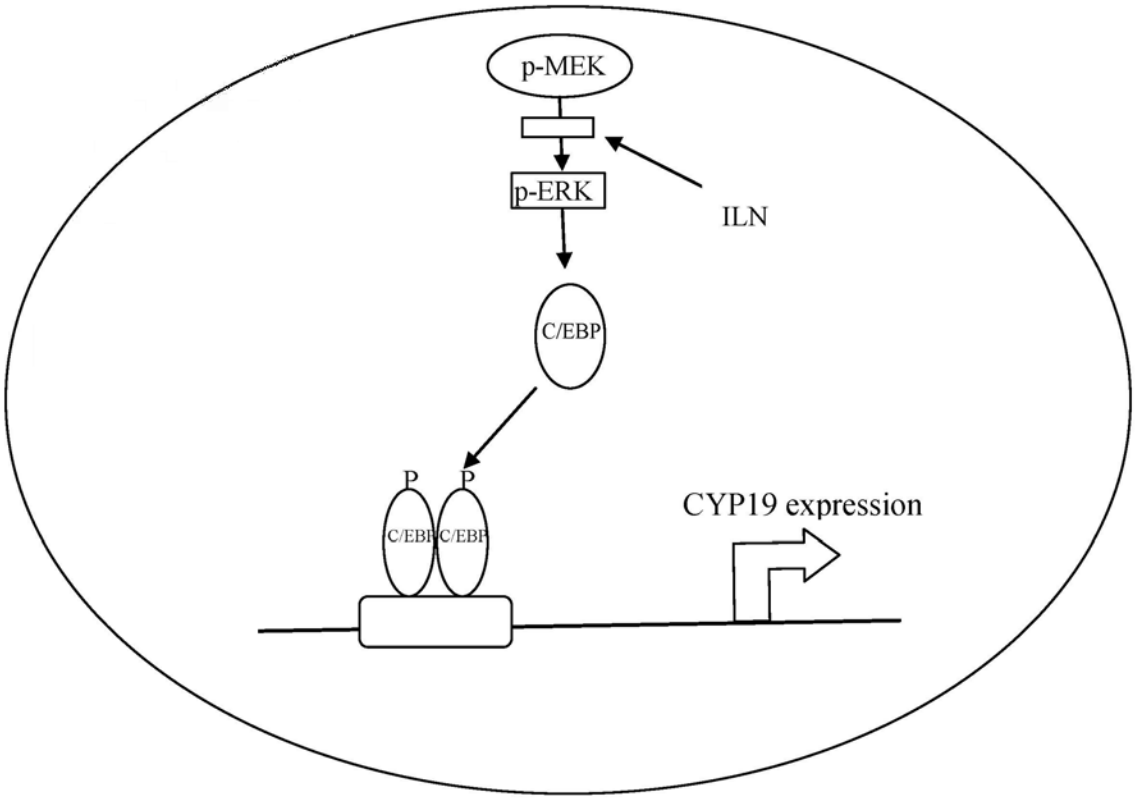



**Fig.8.1** Potential inhibitory pathway of ILN on CYP19 expression in MCF-7 cells

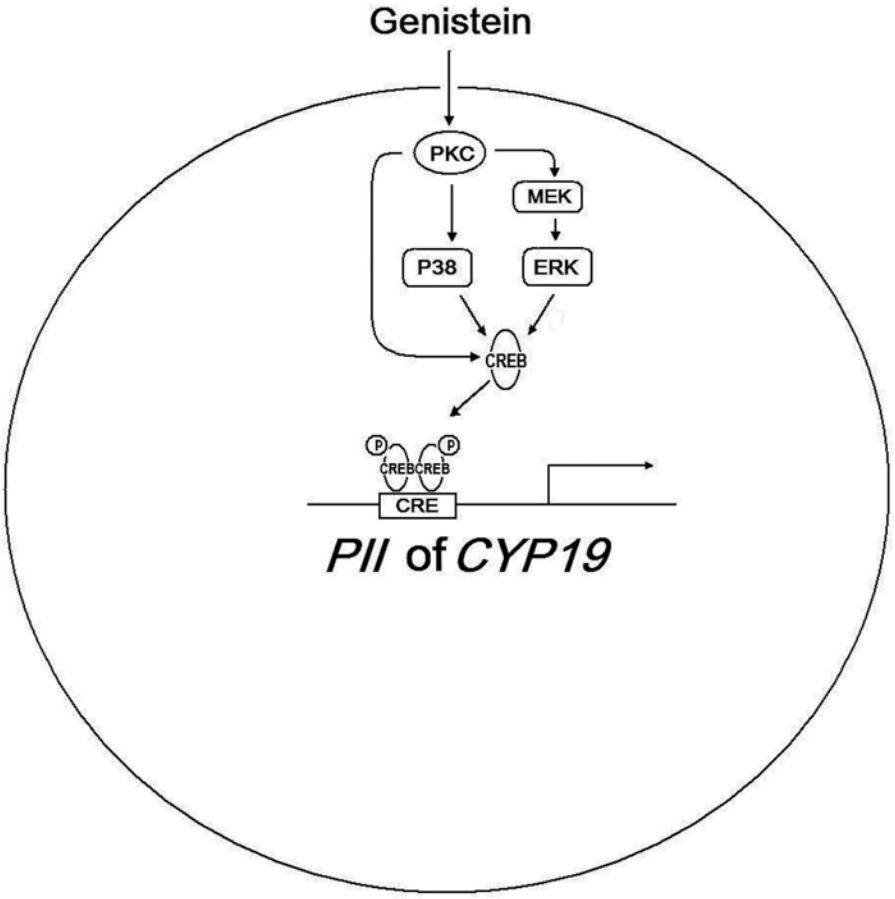



**Fig.8.2** Signaling pathways of genistein inducing CYP19 expression in HepG2 cells.

# BIBLIOGRAPHY

American Cancer Society, Facts and Figures, 2009.

Abdelgadir, S. E., J. A. Resko, S. R. Ojeda, E. D. Lephart, M. J. McPhaul and C. E. Roselli (1994). "Androgens regulate aromatase cytochrome P450 messenger ribonucleic acid in rat brain." Endocrinology **135**(1): 395-401.

Allred, C. D., K. F. Allred, Y. H. Ju, S. M. Virant and W. G. Helferich (2001). "Soy diets containing varying amounts of genistein stimulate growth of estrogen-dependent (MCF-7) tumors in a dose-dependent manner." Cancer Res **61**(13): 5045-50.

Aranganathan, S., J. Panneer Selvam and N. Nalini (2008). "Hesperetin exerts dose dependent chemopreventive effect against 1,2-dimethyl hydrazine induced rat colon carcinogenesis." Invest New Drugs.

Augustowska, K., E. L. Gregoraszczuk, T. Milewicz, J. Krzysiek, A. Grochowalski and R. Chrzaszcz (2003). "Effects of dioxin (2,3,7,8-TCDD) and PCDDs/PCDFs congeners mixture on steroidogenesis in human placenta tissue culture." Endocr Regul **37**(1): 11-9.

Baba, M., R. Asano, I. Takigami, T. Takahashi, M. Ohmura, Y. Okada, H. Sugimoto, T. Arika, H. Nishino and T. Okuyama (2002). "Studies on cancer chemoprevention by traditional folk medicines XXV. Inhibitory effect of isoliquiritigenin on azoxymethane-induced murine colon aberrant crypt focus formation and carcinogenesis." Biol Pharm Bull **25**(2): 247-50.

Benavente-Garcia, O., J. Castillo, M. Alcaraz, V. Vicente, J. A. Del Rio and A. Ortuno (2007). "Beneficial action of Citrus flavonoids on multiple cancer-related biological pathways." Curr Cancer Drug Targets **7**(8): 795-809.

Birnbaum, L. S. and J. Tuomisto (2000). "Non-carcinogenic effects of TCDD in animals." Food Addit Contam **17**(4): 275-88.

Bock, K. W., H. Gschaidmeier, H. Heel, T. Lehmkoster, P. A. Munzel and B. S. Bock-Hennig (1999). "Functions and transcriptional regulation of PAH-inducible human UDP-glucuronosyltransferases." Drug Metab Rev **31**(2):

411-22.

Borradaile, N. M., L. E. de Dreu, L. J. Wilcox, J. Y. Edwards and M. W. Huff (2002). "Soya phytoestrogens, genistein and daidzein, decrease apolipoprotein B secretion from HepG2 cells through multiple mechanisms." Biochem J **366**(Pt 2): 531-9.

Bourguiba, S., C. Genissel, S. Lambard, H. Bouraima and S. Carreau (2003). "Regulation of aromatase gene expression in Leydig cells and germ cells." J Steroid Biochem Mol Biol **86**(3-5): 335-43.

Boverhof, D. R., J. C. Kwekel, D. G. Humes, L. D. Burgoon and T. R. Zacharewski (2006). "Dioxin induces an estrogen-like, estrogen receptor-dependent gene expression response in the murine uterus." Mol Pharmacol **69**(5): 1599-606.

Brodie, A., Q. Lu and J. Nakamura (1997). "Aromatase in the normal breast and breast cancer." J Steroid Biochem Mol Biol **61**(3-6): 281-6.

Brooks, J. D. and L. U. Thompson (2005). "Mammalian lignans and genistein decrease the activities of aromatase and 17beta-hydroxysteroid dehydrogenase in MCF-7 cells." J Steroid Biochem Mol Biol **94**(5): 461-7.

Brown, N. M., P. A. Manzolillo, J. X. Zhang, J. Wang and C. A. Lamartiniere (1998). "Prenatal TCDD and predisposition to mammary cancer in the rat." Carcinogenesis **19**(9): 1623-9.

Brueggemeier, R. W., X. Gu, J. A. Mobley, S. Joomprabutra, A. S. Bhat and J. L. Whetstone (2001). "Effects of phytoestrogens and synthetic combinatorial libraries on aromatase, estrogen biosynthesis, and metabolism." Ann N Y Acad Sci **948**: 51-66.

Butt, A. J., C. E. Caldon, C. M. McNeil, A. Swarbrick, E. A. Musgrove and R. L. Sutherland (2008). "Cell cycle machinery: links with genesis and treatment of breast cancer." Adv Exp Med Biol **630**: 189-205.

Cai, H., U. Smola, V. Wixler, I. Eisenmann-Tappe, M. T. Diaz-Meco, J. Moscat, U. Rapp and G. M. Cooper (1997). "Role of diacylglycerol-regulated protein kinase C isotypes in growth factor activation of the Raf-1 protein kinase." Mol Cell Biol **17**(2): 732-41.

Chen, S. (1998). "Aromatase and breast cancer." Front Biosci **3**: d922-33.

Chen, S. (2002). "Modulation of aromatase activity and expression by environmental chemicals." Front Biosci **7**: d1712-9.

Chen, S., T. Itoh, K. Wu, D. Zhou and C. Yang (2002). "Transcriptional regulation of aromatase expression in human breast tissue." J Steroid Biochem Mol Biol **83**(1-5): 93-9.

Chen, S., F. Zhang, M. A. Sherman, I. Kijima, M. Cho, Y. C. Yuan, Y. Toma, Y. Osawa, D. Zhou and E. T. Eng (2003). "Structure-function studies of aromatase and its inhibitors: a progress report." J Steroid Biochem Mol Biol **86**(3-5): 231-7.

Chen, S., D. Zhou, T. Okubo, Y. C. Kao and C. Yang (1999). "Breast tumor aromatase: functional role and transcriptional regulation." Endocr Relat Cancer **6**(2): 149-56.

Chen, S. A., M. J. Besman, R. S. Sparkes, S. Zollman, I. Klisak, T. Mohandas, P. F. Hall and J. E. Shively (1988). "Human aromatase: cDNA cloning, Southern blot analysis, and assignment of the gene to chromosome 15." DNA **7**(1): 27-38.

Chen, Y., M. D. Gammon, S. L. Teitelbaum, J. A. Britton, M. B. Terry, S. Shantakumar, S. M. Eng, Q. Wang, I. Gurvich, A. I. Neugut, R. M. Santella and H. Ahsan (2008). "Estrogen-biosynthesis gene CYP17 and its interactions with reproductive, hormonal and lifestyle factors in breast cancer risk: results from the Long Island Breast Cancer Study Project." Carcinogenesis **29**(4): 766-71.

Choi, E. J. (2007). "Hesperetin induced G1-phase cell cycle arrest in human breast cancer MCF-7 cells: involvement of CDK4 and p21." Nutr Cancer **59**(1): 115-9.

Choi, E. J., G. D. Kim, K. M. Chee and G. H. Kim (2006). "Effects of hesperetin on vessel structure formation in mouse embryonic stem (mES) cells." Nutrition **22**(9): 947-51.

Chung, S. Y., M. K. Sung, N. H. Kim, J. O. Jang, E. J. Go and H. J. Lee (2005). "Inhibition of P-glycoprotein by natural products in human breast cancer cells." Arch Pharm Res **28**(7): 823-8.

Ciocca, D. R. and M. A. Fanelli (1997). "Estrogen receptors and cell proliferation in

breast cancer." Trends Endocrinol Metab **8**(8): 313-21.

Clyne, C. D., A. Kovacic, C. J. Speed, J. Zhou, V. Pezzi and E. R. Simpson (2004). "Regulation of aromatase expression by the nuclear receptor LRH-1 in adipose tissue." Mol Cell Endocrinol **215**(1-2): 39-44.

Clyne, C. D., C. J. Speed, J. Zhou and E. R. Simpson (2002). "Liver receptor homologue-1 (LRH-1) regulates expression of aromatase in preadipocytes." J Biol Chem **277**(23): 20591-7.

Colditz, G. A. (1999). "Hormones and breast cancer: evidence and implications for consideration of risks and benefits of hormone replacement therapy." J Womens Health **8**(3): 347-57.

Creveling, C. R. (2003). "Reduced COMT activity as a possible environmental risk factor for breast cancer. Opinion." Neurotox Res **5**(7): 473-4.

Dai, Y. and S. Grant (2007). "Targeting multiple arms of the apoptotic regulatory machinery." Cancer Res **67**(7): 2908-11.

Dalton, S. and R. Treisman (1992). "Characterization of SAP-1, a protein recruited by serum response factor to the c-fos serum response element." Cell **68**(3): 597-612.

Dasmahapatra, A. K., B. A. Wimpee, A. L. Trewin, C. F. Wimpee, J. K. Ghorai and R. J. Hutz (2000). "Demonstration of 2,3,7,8-tetrachlorodibenzo-p-dioxin attenuation of P450 steroidogenic enzyme mRNAs in rat granulosa cell in vitro by competitive reverse transcriptase-polymerase chain reaction assay." Mol Cell Endocrinol **164**(1-2): 5-18.

Davis, R. J. (1993). "The mitogen-activated protein kinase signal transduction pathway." J Biol Chem **268**(20): 14553-6.

De Vivo, I., S. E. Hankinson, L. Li, G. A. Colditz and D. J. Hunter (2002). "Association of CYP1B1 polymorphisms and breast cancer risk." Cancer Epidemiol Biomarkers Prev **11**(5): 489-92.

Desaulniers, D., K. Leingartner, J. Russo, G. Perkins, B. G. Chittim, M. C. Archer, M. Wade and J. Yang (2001). "Modulatory effects of neonatal exposure to TCDD, or a mixture of PCBs, p,p'-DDT, and p-p'-DDE, on methylnitrosourea-induced

mammary tumor development in the rat." Environ Health Perspect **109**(7): 739-47.

Diaz-Cruz, E. S., C. L. Shapiro and R. W. Brueggemeier (2005). "Cyclooxygenase inhibitors suppress aromatase expression and activity in breast cancer cells." J Clin Endocrinol Metab **90**(5): 2563-70.

Dickson, R. B., A. Kasid, K. K. Huff, S. E. Bates, C. Knabbe, D. Bronzert, E. P. Gelmann and M. E. Lippman (1987). "Activation of growth factor secretion in tumorigenic states of breast cancer induced by 17 beta-estradiol or v-Ha-ras oncogene." Proc Natl Acad Sci U S A **84**(3): 837-41.

Doostdar, H., M. D. Burke and R. T. Mayer (2000). "Bioflavonoids: selective substrates and inhibitors for cytochrome P450 CYP1A and CYP1B1." Toxicology **144**(1-3): 31-8.

Drenth, H. J., C. A. Bouwman, W. Seinen and M. Van den Berg (1998). "Effects of some persistent halogenated environmental contaminants on aromatase (CYP19) activity in the human choriocarcinoma cell line JEG-3." Toxicol Appl Pharmacol **148**(1): 50-5.

Edmunds, K. M., A. C. Holloway, D. J. Crankshaw, S. K. Agarwal and W. G. Foster (2005). "The effects of dietary phytoestrogens on aromatase activity in human endometrial stromal cells." Reprod Nutr Dev **45**(6): 709-20.

Eisenbrand, G., F. Hippe, S. Jakobs and S. Muehlbeyer (2004). "Molecular mechanisms of indirubin and its derivatives: novel anticancer molecules with their origin in traditional Chinese phytomedicine." J Cancer Res Clin Oncol **130**(11): 627-35.

Ellem, S. J., J. F. Schmitt, J. S. Pedersen, M. Frydenberg and G. P. Risbridger (2004). "Local aromatase expression in human prostate is altered in malignancy." J Clin Endocrinol Metab **89**(5): 2434-41.

Eng, E. T., D. Williams, U. Mandava, N. Kirma, R. R. Tekmal and S. Chen (2001). "Suppression of aromatase (estrogen synthetase) by red wine phytochemicals." Breast Cancer Res Treat **67**(2): 133-46.

Eng, E. T., J. Ye, D. Williams, S. Phung, R. E. Moore, M. K. Young, U. Gruntmanis,

G. Braunstein and S. Chen (2003). "Suppression of estrogen biosynthesis by procyanidin dimers in red wine and grape seeds." Cancer Res **63**(23): 8516-22.

Erlund, I., E. Meririnne, G. Alfthan and A. Aro (2001). "Plasma kinetics and urinary excretion of the flavanones naringenin and hesperetin in humans after ingestion of orange juice and grapefruit juice." J Nutr **131**(2): 235-41.

Esteban, J. M., Z. Warsi, M. Haniu, P. Hall, J. E. Shively and S. Chen (1992). "Detection of intratumoral aromatase in breast carcinomas. An immunohistochemical study with clinicopathologic correlation." Am J Pathol **140**(2): 337-43.

Foster, J. S., D. C. Henley, A. Bukovsky, P. Seth and J. Wimalasena (2001). "Multifaceted regulation of cell cycle progression by estrogen: regulation of Cdk inhibitors and Cdc25A independent of cyclin D1-Cdk4 function." Mol Cell Biol **21**(3): 794-810.

Foster, W. G. (2008). "Endocrine toxicants including 2,3,7,8-terachlorodibenzo-p-dioxin (TCDD) and dioxin-like chemicals and endometriosis: is there a link?" J Toxicol Environ Health B Crit Rev **11**(3-4): 177-87.

Gallo, D., C. Ferlini, M. Fabrizi, S. Prislei and G. Scambia (2006). "Lack of stimulatory activity of a phytoestrogen-containing soy extract on the growth of breast cancer tumors in mice." Carcinogenesis **27**(7): 1404-9.

Galmiche, G., S. Corvaisier and M. L. Kottler (2006). "Aromatase gene expression and regulation in the female rat pituitary." Ann N Y Acad Sci **1070**: 286-92.

Gao, K., S. M. Henning, Y. Niu, A. A. Youssefian, N. P. Seeram, A. Xu and D. Heber (2006). "The citrus flavonoid naringenin stimulates DNA repair in prostate cancer cells." J Nutr Biochem **17**(2): 89-95.

Garrington, T. P. and G. L. Johnson (1999). "Organization and regulation of mitogen-activated protein kinase signaling pathways." Curr Opin Cell Biol **11**(2): 211-8.

Ghosh, S., Y. Wu, R. Li and Y. Hu (2005). "Jun proteins modulate the ovary-specific promoter of aromatase gene in ovarian granulosa cells via a cAMP-responsive

element." Oncogene **24**(13): 2236-46.

Gille, H., A. D. Sharrocks and P. E. Shaw (1992). "Phosphorylation of transcription factor p62TCF by MAP kinase stimulates ternary complex formation at c-fos promoter." Nature **358**(6385): 414-7.

Goth-Goldstein, R., M. R. Stampfer, C. A. Erdmann and M. Russell (2000). "Interindividual variation in CYP1A1 expression in breast tissue and the role of genetic polymorphism." Carcinogenesis **21**(11): 2119-22.

Graham-Lorence, S., B. Amarneh, R. E. White, J. A. Peterson and E. R. Simpson (1995). "A three-dimensional model of aromatase cytochrome P450." Protein Sci **4**(6): 1065-80.

Grana, X. and E. P. Reddy (1995). "Cell cycle control in mammalian cells: role of cyclins, cyclin dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CKIs)." Oncogene **11**(2): 211-9.

Grimm, S. L. and J. M. Rosen (2003). "The role of C/EBPbeta in mammary gland development and breast cancer." J Mammary Gland Biol Neoplasia **8**(2): 191-204.

Grube, B. J., E. T. Eng, Y. C. Kao, A. Kwon and S. Chen (2001). "White button mushroom phytochemicals inhibit aromatase activity and breast cancer cell proliferation." J Nutr **131**(12): 3288-93.

Guillemette, C., R. C. Millikan, B. Newman and D. E. Housman (2000). "Genetic polymorphisms in uridine diphospho-glucuronosyltransferase 1A1 and association with breast cancer among African Americans." Cancer Res **60**(4): 950-6.

Guthrie, N. and K. K. Carroll (1998). "Inhibition of mammary cancer by citrus flavonoids." Adv Exp Med Biol **439**: 227-36.

Hamajima, N., K. Matsuo, K. Tajima, M. Mizutani, H. Iwata, T. Iwase, S. Miura, H. Oya and Y. Obata (2001). "Limited association between a catechol-O-methyltransferase (COMT) polymorphism and breast cancer risk in Japan." Int J Clin Oncol **6**(1): 13-8.

Hanagiri, T., T. Nozoe, T. Yoshimatsu, M. Mizukami, Y. Ichiki, M. Sugaya, M.

Yasuda, M. Takenoyama, K. Sugio, H. Yasuda and K. Yasumoto (2008). "Surgical treatment for chest wall invasion due to the local recurrence of breast cancer." Breast Cancer **15**(4): 298-302.

Harada, H. and S. Grant (2003). "Apoptosis regulators." Rev Clin Exp Hematol **7**(2): 117-38.

Harada, N., N. Yoshimura and S. Honda (2003). "Unique regulation of expression of human aromatase in the placenta." J Steroid Biochem Mol Biol **86**(3-5): 327-34.

Harmon, A. W. and Y. M. Patel (2004). "Naringenin inhibits glucose uptake in MCF-7 breast cancer cells: a mechanism for impaired cellular proliferation." Breast Cancer Res Treat **85**(2): 103-10.

Hector, S. and J. H. Prehn (2009). "Apoptosis signaling proteins as prognostic biomarkers in colorectal cancer: a review." Biochim Biophys Acta **1795**(2): 117-29.

Hernandez, R. K., H. T. Sorensen, J. Jacobsen, L. Pedersen and T. L. Lash (2008). "Tamoxifen treatment in Danish breast cancer patients and 5-year risk of arterial atherosclerotic events: a null association." Cancer Epidemiol Biomarkers Prev **17**(9): 2509-11.

Hiyama, H., A. Iavarone, J. LaBaer and S. A. Reeves (1997). "Regulated ectopic expression of cyclin D1 induces transcriptional activation of the cdk inhibitor p21 gene without altering cell cycle progression." Oncogene **14**(21): 2533-42.

Honda, S., N. Harada and Y. Takagi (1996). "The alternative exons 1 of the mouse aromatase cytochrome P-450 gene." Biochim Biophys Acta **1305**(3): 145-50.

Hsieh, C. Y., R. C. Santell, S. Z. Haslam and W. G. Helferich (1998). "Estrogenic effects of genistein on the growth of estrogen receptor-positive human breast cancer (MCF-7) cells in vitro and in vivo." Cancer Res **58**(17): 3833-8.

Huang, C. S., H. D. Chern, K. J. Chang, C. W. Cheng, S. M. Hsu and C. Y. Shen (1999). "Breast cancer risk associated with genotype polymorphism of the estrogen-metabolizing genes CYP17, CYP1A1, and COMT: a multigenic study on cancer susceptibility." Cancer Res **59**(19): 4870-5.

Huang, Z., M. J. Fasco, H. L. Figge, K. Keyomarsi and L. S. Kaminsky (1996).

"Expression of cytochromes P450 in human breast tissue and tumors." Drug Metab Dispos **24**(8): 899-905.

Ii, T., Y. Satomi, D. Katoh, J. Shimada, M. Baba, T. Okuyama, H. Nishino and N. Kitamura (2004). "Induction of cell cycle arrest and p21(CIP1/WAF1) expression in human lung cancer cells by isoliquiritigenin." Cancer Lett **207**(1): 27-35.

Ikeda, M., J. Kurebayashi, H. Sonoo, A. Miyake, T. Nomura, Y. Yamamoto, S. Shiiki, K. Nakashima and K. Tanaka (2008). "[Efficacy of sequential treatment with anastrozole following exemestane compared with exemestane following anastrozole in patients with metastatic breast cancer]." Gan To Kagaku Ryoho **35**(2): 261-5.

Ikeda, M., T. Mitsui, K. Setani, M. Tamura, M. Kakeyama, H. Sone, C. Tohyama and T. Tomita (2005). "In utero and lactational exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin in rats disrupts brain sexual differentiation." Toxicol Appl Pharmacol **205**(1): 98-105.

Izumi, T., M. K. Piskula, S. Osawa, A. Obata, K. Tobe, M. Saito, S. Kataoka, Y. Kubota and M. Kikuchi (2000). "Soy isoflavone aglycones are absorbed faster and in higher amounts than their glucosides in humans." J Nutr **130**(7): 1695-9.

James, V. H., J. M. McNeill, L. C. Lai, C. J. Newton, M. W. Ghilchik and M. J. Reed (1987). "Aromatase activity in normal breast and breast tumor tissues: in vivo and in vitro studies." Steroids **50**(1-3): 269-79.

Jelovac, D., L. Macedo, V. Handratta, B. J. Long, O. G. Goloubeva, J. N. Ingle and A. M. Brodie (2004). "Effects of exemestane and tamoxifen in a postmenopausal breast cancer model." Clin Cancer Res **10**(21): 7375-81.

Jeong, H. J., Y. G. Shin, I. H. Kim and J. M. Pezzuto (1999). "Inhibition of aromatase activity by flavonoids." Arch Pharm Res **22**(3): 309-12.

Ji, Y., J. Olson, J. Zhang, M. Hildebrandt, L. Wang, J. Ingle, Z. Fredericksen, T. Sellers, W. Miller, J. M. Dixon, H. Brauch, M. Eichelbaum, C. Justenhoven, U. Hamann, Y. Ko, T. Bruning, J. Chang-Claude, S. Wang-Gohrke, D. Schaid and R. Weinshilboum (2008). "Breast cancer risk reduction and membrane-bound

catechol O-methyltransferase genetic polymorphisms." Cancer Res **68**(14): 5997-6005.

Jin, Y. R., X. H. Han, Y. H. Zhang, J. J. Lee, Y. Lim, T. J. Kim, H. S. Yoo and Y. P. Yun (2008). "Hesperetin, a bioflavonoid, inhibits rat aortic vascular smooth muscle cells proliferation by arresting cell cycle." J Cell Biochem **104**(1): 1-14.

Jo, E. H., H. D. Hong, N. C. Ahn, J. W. Jung, S. R. Yang, J. S. Park, S. H. Kim, Y. S. Lee and K. S. Kang (2004). "Modulations of the Bcl-2/Bax family were involved in the chemopreventive effects of licorice root (Glycyrrhiza uralensis Fisch) in MCF-7 human breast cancer cell." J Agric Food Chem **52**(6): 1715-9.

Jo, E. H., S. H. Kim, J. C. Ra, S. R. Kim, S. D. Cho, J. W. Jung, S. R. Yang, J. S. Park, J. W. Hwang, O. I. Aruoma, T. Y. Kim, Y. S. Lee and K. S. Kang (2005). "Chemopreventive properties of the ethanol extract of chinese licorice (Glycyrrhiza uralensis) root: induction of apoptosis and G1 cell cycle arrest in MCF-7 human breast cancer cells." Cancer Lett **230**(2): 239-47.

Ju, Y. H., K. F. Allred, C. D. Allred and W. G. Helferich (2006). "Genistein stimulates growth of human breast cancer cells in a novel, postmenopausal animal model, with low plasma estradiol concentrations." Carcinogenesis **27**(6): 1292-9.

Jung, J. I., E. Chung, M. R. Seon, H. K. Shin, E. J. Kim, S. S. Lim, W. Y. Chung, K. K. Park and J. H. Park (2006). "Isoliquiritigenin (ISL) inhibits ErbB3 signaling in prostate cancer cells." Biofactors **28**(3-4): 159-68.

Kao, Y. C., C. Zhou, M. Sherman, C. A. Laughton and S. Chen (1998). "Molecular basis of the inhibition of human aromatase (estrogen synthetase) by flavone and isoflavone phytoestrogens: A site-directed mutagenesis study." Environ Health Perspect **106**(2): 85-92.

Karin, M. and E. Shaulian (2001). "AP-1: linking hydrogen peroxide and oxidative stress to the control of cell proliferation and death." IUBMB Life **52**(1-2): 17-24.

Key, T., P. Appleby, I. Barnes and G. Reeves (2002). "Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies." J Natl Cancer Inst **94**(8): 606-16.

Kijima, I., S. Phung, G. Hur, S. L. Kwok and S. Chen (2006). "Grape seed extract is an aromatase inhibitor and a suppressor of aromatase expression." Cancer Res **66**(11): 5960-7.

Kijima, I., J. Ye, C. Glackin and S. Chen (2008). "CCAAT/enhancer binding protein delta up-regulates aromatase promoters I.3/II in breast cancer epithelial cells." Cancer Res **68**(11): 4455-64.

Kim, R. (2005). "Unknotting the roles of Bcl-2 and Bcl-xL in cell death." Biochem Biophys Res Commun **333**(2): 336-43.

Kinoshita, Y. and S. Chen (2003). "Induction of aromatase (CYP19) expression in breast cancer cells through a nongenomic action of estrogen receptor alpha." Cancer Res **63**(13): 3546-55.

Klinge, C. M., J. L. Bowers, P. C. Kulakosky, K. K. Kamboj and H. I. Swanson (1999). "The aryl hydrocarbon receptor (AHR)/AHR nuclear translocator (ARNT) heterodimer interacts with naturally occurring estrogen response elements." Mol Cell Endocrinol **157**(1-2): 105-19.

Knerr, S. and D. Schrenk (2006). "Carcinogenicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in experimental models." Mol Nutr Food Res **50**(10): 897-907.

Kolch, W., G. Heidecker, G. Kochs, R. Hummel, H. Vahidi, H. Mischak, G. Finkenzeller, D. Marme and U. R. Rapp (1993). "Protein kinase C alpha activates RAF-1 by direct phosphorylation." Nature **364**(6434): 249-52.

Kudachadkar, R. and R. M. O'Regan (2005). "Aromatase inhibitors as adjuvant therapy for postmenopausal patients with early stage breast cancer." CA Cancer J Clin **55**(3): 145-63.

Kuiper, G. G., J. G. Lemmen, B. Carlsson, J. C. Corton, S. H. Safe, P. T. van der Saag, B. van der Burg and J. A. Gustafsson (1998). "Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta." Endocrinology **139**(10): 4252-63.

Lai, K. P., M. H. Wong and C. K. Wong (2005). "Effects of TCDD in modulating the expression of Sertoli cell secretory products and markers for cell-cell

interaction." Toxicology **206**(1): 111-23.

Lamon-Fava, S. and D. Micherone (2004). "Regulation of apoA-I gene expression: mechanism of action of estrogen and genistein." J Lipid Res **45**(1): 106-12.

Lamon-Fava, S., J. M. Ordovas and E. J. Schaefer (1999). "Estrogen increases apolipoprotein (apo) A-I secretion in hep G2 cells by modulating transcription of the apo A-I gene promoter." Arterioscler Thromb Vasc Biol **19**(12): 2960-5.

Le Bail, J. C., C. Pouget, C. Fagnere, J. P. Basly, A. J. Chulia and G. Habrioux (2001). "Chalcones are potent inhibitors of aromatase and 17beta-hydroxysteroid dehydrogenase activities." Life Sci **68**(7): 751-61.

Lea, C. K., U. Sarma and A. M. Flanagan (1999). "Macrophage colony stimulating-factor transcripts are differentially regulated in rat bone-marrow by gender hormones." Endocrinology **140**(1): 273-9.

Lee, H. O. and Y. Y. Sheen (1997). "Estrogen modulation of human breast cancer cell growth." Arch Pharm Res **20**(6): 566-71.

Lee, Y., R. A. Renaud, T. C. Friedrich and J. Gorski (1998). "Estrogen causes cell death of estrogen receptor stably transfected cells via apoptosis." J Steroid Biochem Mol Biol **67**(4): 327-32.

Leung, H. Y., Y. Wang and L. K. Leung (2007). "Differential effect of over-expressing UGT1A1 and CYP1A1 on xenobiotic assault in MCF-7 cells." Toxicology **242**(1-3): 153-9.

Leung, L. K. and T. T. Wang (1999). "Differential effects of chemotherapeutic agents on the Bcl-2/Bax apoptosis pathway in human breast cancer cell line MCF-7." Breast Cancer Res Treat **55**(1): 73-83.

Leung, L. K. and T. T. Wang (1999). "Paradoxical regulation of Bcl-2 family proteins by 17beta-oestradiol in human breast cancer cells MCF-7." Br J Cancer **81**(3): 387-92.

Leung, L. K., Y. M. Yuen, H. Y. Leung and Y. Wang (2005). "Dietary soya isoflavones and breast carcinogenesis: a perspective from a cell-culture model." Nutr Res Rev **18**(2): 202-11.

Li, H., P. Gade, W. Xiao and D. V. Kalvakolanu (2007). "The interferon signaling

network and transcription factor C/EBP-beta." Cell Mol Immunol **4**(6): 407-18.

Li, J. J. and S. A. Li (1987). "Estrogen carcinogenesis in Syrian hamster tissues: role of metabolism." Fed Proc **46**(5): 1858-63.

Liehr, J. G. (2000). "Is estradiol a genotoxic mutagenic carcinogen?" Endocr Rev **21**(1): 40-54.

Liehr, J. G., A. A. Ulubelen and H. W. Strobel (1986). "Cytochrome P-450-mediated redox cycling of estrogens." J Biol Chem **261**(36): 16865-70.

Lindahl, B., E. Andolf, C. Ingvar, J. Ranstam and R. Willen (2008). "Adjuvant tamoxifen in breast cancer patients affects the endometrium by time, an effect remaining years after end of treatment and results in an increased frequency of endometrial carcinoma." Anticancer Res **28**(2B): 1259-62.

Liu, L., D. M. Xu and Y. Y. Cheng (2008). "Distinct effects of naringenin and hesperetin on nitric oxide production from endothelial cells." J Agric Food Chem **56**(3): 824-9.

Lonning, P. E. and J. Geisler (2008). "Experience with exemestane in the treatment of early and advanced breast cancer." Expert Opin Drug Metab Toxicol **4**(7): 987-97.

Lonning, P. E., J. Geisler, D. C. Johannessen and D. Ekse (1997). "Plasma estrogen suppression with aromatase inhibitors evaluated by a novel, sensitive assay for estrone sulphate." J Steroid Biochem Mol Biol **61**(3-6): 255-60.

Maggiolini, M., G. Statti, A. Vivacqua, S. Gabriele, V. Rago, M. Loizzo, F. Menichini and S. Amdo (2002). "Estrogenic and antiproliferative activities of isoliquiritigenin in MCF7 breast cancer cells." J Steroid Biochem Mol Biol **82**(4-5): 315-22.

Mahendroo, M. S., C. R. Mendelson and E. R. Simpson (1993). "Tissue-specific and hormonally controlled alternative promoters regulate aromatase cytochrome P450 gene expression in human adipose tissue." J Biol Chem **268**(26): 19463-70.

Mai, Z., G. L. Blackburn and J. R. Zhou (2007). "Soy phytochemicals synergistically enhance the preventive effect of tamoxifen on the growth of estrogen-dependent

human breast carcinoma in mice." Carcinogenesis **28**(6): 1217-23.

Mantena, S. K., S. D. Sharma and S. K. Katiyar (2006). "Berberine, a natural product, induces G1-phase cell cycle arrest and caspase-3-dependent apoptosis in human prostate carcinoma cells." Mol Cancer Ther **5**(2): 296-308.

Manthey, J. A. and N. Guthrie (2002). "Antiproliferative activities of citrus flavonoids against six human cancer cell lines." J Agric Food Chem **50**(21): 5837-43.

Margreiter, R. (1980). "[The antiestrogen tamoxifen in advanced breast cancer (author's transl)]." Langenbecks Arch Chir **351**(4): 249-62.

Matsuoka, M., K. Tani and S. Asano (1998). "Interferon-alpha-induced G1 phase arrest through up-regulated expression of CDK inhibitors, p19Ink4D and p21Cip1 in mouse macrophages." Oncogene **16**(16): 2075-86.

McKay, J. A., W. T. Melvin, A. K. Ah-See, S. W. Ewen, W. F. Greenlee, C. B. Marcus, M. D. Burke and G. I. Murray (1995). "Expression of cytochrome P450 CYP1B1 in breast cancer." FEBS Lett **374**(2): 270-2.

Means, G. D., M. S. Mahendroo, C. J. Corbin, J. M. Mathis, F. E. Powell, C. R. Mendelson and E. R. Simpson (1989). "Structural analysis of the gene encoding human aromatase cytochrome P-450, the enzyme responsible for estrogen biosynthesis." J Biol Chem **264**(32): 19385-91.

Michael, M. D., M. W. Kilgore, K. Morohashi and E. R. Simpson (1995). "Ad4BP/SF-1 regulates cyclic AMP-induced transcription from the proximal promoter (PII) of the human aromatase P450 (CYP19) gene in the ovary." J Biol Chem **270**(22): 13561-6.

Miller, W. R. and J. M. Dixon (2001). "Local endocrine effects of aromatase inhibitors within the breast." J Steroid Biochem Mol Biol **79**(1-5): 93-102.

Miller, W. R., F. Howie and I. Mason (2001). "[Aromatase and breast cancer]." Vopr Onkol **47**(2): 182-6.

Miners, J. O. and P. I. Mackenzie (1991). "Drug glucuronidation in humans." Pharmacol Ther **51**(3): 347-69.

Miranda, C. L., J. F. Stevens, A. Helmrich, M. C. Henderson, R. J. Rodriguez, Y. H.

Yang, M. L. Deinzer, D. W. Barnes and D. R. Buhler (1999). "Antiproliferative and cytotoxic effects of prenylated flavonoids from hops (Humulus lupulus) in human cancer cell lines." Food Chem Toxicol **37**(4): 271-85.

Moran, F. M., A. J. Conley, C. J. Corbin, E. Enan, C. VandeVoort, J. W. Overstreet and B. L. Lasley (2000). "2,3,7,8-tetrachlorodibenzo-p-dioxin decreases estradiol production without altering the enzyme activity of cytochrome P450 aromatase of human luteinized granulosa cells in vitro." Biol Reprod **62**(4): 1102-8.

Myllymaki, S., T. Haavisto, M. Vainio, J. Toppari and J. Paranko (2005). "In vitro effects of diethylstilbestrol, genistein, 4-tert-butylphenol, and 4-tert-octylphenol on steroidogenic activity of isolated immature rat ovarian follicles." Toxicol Appl Pharmacol **204**(1): 69-80.

Myllymaki, S. A., T. E. Haavisto, L. J. Brokken, M. Viluksela, J. Toppari and J. Paranko (2005). "In utero and lactational exposure to TCDD; steroidogenic outcomes differ in male and female rat pups." Toxicol Sci **88**(2): 534-44.

Nebert, D. W., T. P. Dalton, A. B. Okey and F. J. Gonzalez (2004). "Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer." J Biol Chem **279**(23): 23847-50.

Nkondjock, A. and P. Ghadirian (2005). "[Risk factors and risk reduction of breast cancer]." Med Sci (Paris) **21**(2): 175-80.

Ohtake, F., K. Takeyama, T. Matsumoto, H. Kitagawa, Y. Yamamoto, K. Nohara, C. Tohyama, A. Krust, J. Mimura, P. Chambon, J. Yanagisawa, Y. Fujii-Kuriyama and S. Kato (2003). "Modulation of oestrogen receptor signalling by association with the activated dioxin receptor." Nature **423**(6939): 545-50.

Okayama, H. (1994). "[Cell cycle control in mammalian cells]." Seikagaku **66**(2): 126-36.

Pasqualini, J. R., G. Chetrite, C. Blacker, M. C. Feinstein, L. Delalonde, M. Talbi and C. Maloche (1996). "Concentrations of estrone, estradiol, and estrone sulfate and evaluation of sulfatase and aromatase activities in pre- and postmenopausal breast cancer patients." J Clin Endocrinol Metab **81**(4): 1460-4.

Pesonen, S. A., T. E. Haavisto, M. Viluksela, J. Toppari and J. Paranko (2006). "Effects of in utero and lactational exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on rat follicular steroidogenesis." Reprod Toxicol **22**(3): 521-8.

Pike, M. C. and D. V. Spicer (1993). "The chemoprevention of breast cancer by reducing sex steroid exposure: perspectives from epidemiology." J Cell Biochem Suppl **17G**: 26-36.

Po, L. S., Z. Y. Chen, D. S. Tsang and L. K. Leung (2002). "Baicalein and genistein display differential actions on estrogen receptor (ER) transactivation and apoptosis in MCF-7 cells." Cancer Lett **187**(1-2): 33-40.

Prasad, J. C., J. V. Goldstone, C. J. Camacho, S. Vajda and J. J. Stegeman (2007). "Ensemble modeling of substrate binding to cytochromes P450: analysis of catalytic differences between CYP1A orthologs." Biochemistry **46**(10): 2640-54.

Pumford, S. L., M. M. Morton, A. Turkes and K. Griffiths (2002). "Determination of the isoflavonoids genistein and daidzein in biological samples by gas chromatography-mass spectrometry." Ann Clin Biochem **39**(Pt 3): 281-92.

Recht, A. (2008). "New radiation treatment strategies for early stage breast cancer." Cancer Treat Res **141**: 135-48.

Reed, M. W., R. A. Audisio and L. Wyld (2009). "The role of surgery in the treatment of older women with breast cancer." Clin Oncol (R Coll Radiol) **21**(2): 103-10.

Revich, B., E. Aksel, T. Ushakova, I. Ivanova, N. Zhuchenko, N. Klyuev, B. Brodsky and Y. Sotskov (2001). "Dioxin exposure and public health in Chapaevsk, Russia." Chemosphere **43**(4-7): 951-66.

Rier, S. and W. G. Foster (2002). "Environmental dioxins and endometriosis." Toxicol Sci **70**(2): 161-70.

Roos, P. H. and H. M. Bolt (2005). "Cytochrome P450 interactions in human cancers: new aspects considering CYP1B1." Expert Opin Drug Metab Toxicol **1**(2): 187-202.

Rozek, D. and G. P. Pfeifer (1993). "In vivo protein-DNA interactions at the c-jun

promoter: preformed complexes mediate the UV response." Mol Cell Biol **13**(9): 5490-9.

Saintot, M., C. Malaveille, A. Hautefeuille and M. Gerber (2004). "Interaction between genetic polymorphism of cytochrome P450-1B1 and environmental pollutants in breast cancer risk." Eur J Cancer Prev **13**(1): 83-6.

Santner, S. J., S. Chen, D. Zhou, Z. Korsunsky, J. Martel and R. J. Santen (1993). "Effect of androstenedione on growth of untransfected and aromatase-transfected MCF-7 cells in culture." J Steroid Biochem Mol Biol **44**(4-6): 611-6.

Scheiber, M. D., J. H. Liu, M. T. Subbiah, R. W. Rebar and K. D. Setchell (2001). "Dietary inclusion of whole soy foods results in significant reductions in clinical risk factors for osteoporosis and cardiovascular disease in normal postmenopausal women." Menopause **8**(5): 384-92.

Sebastian, S. and S. E. Bulun (2001). "A highly complex organization of the regulatory region of the human CYP19 (aromatase) gene revealed by the Human Genome Project." J Clin Endocrinol Metab **86**(10): 4600-2.

Sebastian, S., K. Takayama, M. Shozu and S. E. Bulun (2002). "Cloning and characterization of a novel endothelial promoter of the human CYP19 (aromatase P450) gene that is up-regulated in breast cancer tissue." Mol Endocrinol **16**(10): 2243-54.

Shao, Z. M., M. L. Alpaugh, J. A. Fontana and S. H. Barsky (1998). "Genistein inhibits proliferation similarly in estrogen receptor-positive and negative human breast carcinoma cell lines characterized by P21WAF1/CIP1 induction, G2/M arrest, and apoptosis." J Cell Biochem **69**(1): 44-54.

Shatalova, E. G., V. I. Loginov, E. A. Braga, T. P. Kazubskaia, M. A. Sudomoina, R. L. Blanchard and O. O. Favorova (2006). "[Association of polymorphisms in SULT1A1 and UGT1A1 Genes with breast cancer risk and phenotypes in Russian women]." Mol Biol (Mosk) **40**(2): 263-70.

Shaulian, E. and M. Karin (2002). "AP-1 as a regulator of cell life and death." Nat Cell Biol **4**(5): E131-6.

Shen, Y., J. P. Costantino and J. Qin (2008). "Tamoxifen chemoprevention treatment and time to first diagnosis of estrogen receptor-negative breast cancer." J Natl Cancer Inst **100**(20): 1448-53.

Sherr, C. J. and J. M. Roberts (1999). "CDK inhibitors: positive and negative regulators of G1-phase progression." Genes Dev **13**(12): 1501-12.

Shields-Botella, J., G. Chetrite, S. Meschi and J. R. Pasqualini (2005). "Effect of nomegestrol acetate on estrogen biosynthesis and transformation in MCF-7 and T47-D breast cancer cells." J Steroid Biochem Mol Biol **93**(1): 1-13.

Shin, A., D. Kang, J. Y. Choi, K. M. Lee, S. K. Park, D. Y. Noh, S. H. Ahn and K. Y. Yoo (2007). "Cytochrome P450 1A1 (CYP1A1) polymorphisms and breast cancer risk in Korean women." Exp Mol Med **39**(3): 361-6.

Shin, E. S., S. Y. Cho, E. H. Lee, S. J. Lee, I. S. Chang and T. R. Lee (2006). "Positive regulation of hepatic carnitine palmitoyl transferase 1A (CPT1A) activities by soy isoflavones and L-carnitine." Eur J Nutr **45**(3): 159-64.

Shin, E. S., H. H. Lee, S. Y. Cho, H. W. Park, S. J. Lee and T. R. Lee (2007). "Genistein downregulates SREBP-1 regulated gene expression by inhibiting site-1 protease expression in HepG2 cells." J Nutr **137**(5): 1127-31.

Shozu, M., Y. Zhao, S. E. Bulun and E. R. Simpson (1998). "Multiple splicing events involved in regulation of human aromatase expression by a novel promoter, I.6." Endocrinology **139**(4): 1610-7.

Shutt, D. A. and R. I. Cox (1972). "Steroid and phyto-oestrogen binding to sheep uterine receptors in vitro." J Endocrinol **52**(2): 299-310.

Simpson, E. R., M. S. Mahendroo, G. D. Means, M. W. Kilgore, M. M. Hinshelwood, S. Graham-Lorence, B. Amarneh, Y. Ito, C. R. Fisher, M. D. Michael and et al. (1994). "Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis." Endocr Rev **15**(3): 342-55.

Simpson, E. R., M. S. Mahendroo, J. E. Nichols and S. E. Bulun (1994). "Aromatase gene expression in adipose tissue: relationship to breast cancer." Int J Fertil Menopausal Stud **39 Suppl 2**: 75-83.

Simpson, E. R., M. D. Michael, V. R. Agarwal, M. M. Hinshelwood, S. E. Bulun and

Y. Zhao (1997). "Cytochromes P450 11: expression of the CYP19 (aromatase) gene: an unusual case of alternative promoter usage." FASEB J **11**(1): 29-36.

Simpson, E. R., Y. Zhao, V. R. Agarwal, M. D. Michael, S. E. Bulun, M. M. Hinshelwood, S. Graham-Lorence, T. Sun, C. R. Fisher, K. Qin and C. R. Mendelson (1997). "Aromatase expression in health and disease." Recent Prog Horm Res **52**: 185-213; discussion 213-4.

Singh, V., N. Rastogi, A. Sinha, A. Kumar, N. Mathur and M. P. Singh (2007). "A study on the association of cytochrome-P450 1A1 polymorphism and breast cancer risk in north Indian women." Breast Cancer Res Treat **101**(1): 73-81.

So, F. V., N. Guthrie, A. F. Chambers and K. K. Carroll (1997). "Inhibition of proliferation of estrogen receptor-positive MCF-7 human breast cancer cells by flavonoids in the presence and absence of excess estrogen." Cancer Lett **112**(2): 127-33.

So, F. V., N. Guthrie, A. F. Chambers, M. Moussa and K. K. Carroll (1996). "Inhibition of human breast cancer cell proliferation and delay of mammary tumorigenesis by flavonoids and citrus juices." Nutr Cancer **26**(2): 167-81.

Sofi, M., M. J. Young, T. Papamakarios, E. R. Simpson and C. D. Clyne (2003). "Role of CRE-binding protein (CREB) in aromatase expression in breast adipose." Breast Cancer Res Treat **79**(3): 399-407.

Spencer, J. P., M. M. Abd-el-Mohsen and C. Rice-Evans (2004). "Cellular uptake and metabolism of flavonoids and their metabolites: implications for their bioactivity." Arch Biochem Biophys **423**(1): 148-61.

Spink, B. C., M. J. Fasco, J. F. Gierthy and D. C. Spink (1998). "12-O-tetradecanoylphorbol-13-acetate upregulates the Ah receptor and differentially alters CYP1B1 and CYP1A1 expression in MCF-7 breast cancer cells." J Cell Biochem **70**(3): 289-96.

Stocco, C., J. Kwintkiewicz and Z. Cai (2007). "Identification of regulatory elements in the Cyp19 proximal promoter in rat luteal cells." J Mol Endocrinol **39**(4): 211-21.

Sun, X. Z., D. Zhou and S. Chen (1997). "Autocrine and paracrine actions of breast

tumor aromatase. A three-dimensional cell culture study involving aromatase transfected MCF-7 and T-47D cells." J Steroid Biochem Mol Biol **63**(1-3): 29-36.

Taioli, E., J. Trachman, X. Chen, P. Toniolo and S. J. Garte (1995). "A CYP1A1 restriction fragment length polymorphism is associated with breast cancer in African-American women." Cancer Res **55**(17): 3757-8.

Takahashi, T., M. Baba, H. Nishino and T. Okuyama (2006). "Cyclooxygenase-2 plays a suppressive role for induction of apoptosis in isoliquiritigenin-treated mouse colon cancer cells." Cancer Lett **231**(2): 319-25.

Takahashi, T., N. Takasuka, M. Iigo, M. Baba, H. Nishino, H. Tsuda and T. Okuyama (2004). "Isoliquiritigenin, a flavonoid from licorice, reduces prostaglandin E2 and nitric oxide, causes apoptosis, and suppresses aberrant crypt foci development." Cancer Sci **95**(5): 448-53.

Tawata, M., K. Aida, T. Noguchi, Y. Ozaki, S. Kume, H. Sasaki, M. Chin and T. Onaya (1992). "Anti-platelet action of isoliquiritigenin, an aldose reductase inhibitor in licorice." Eur J Pharmacol **212**(1): 87-92.

Toda, K., M. Terashima, T. Kawamoto, H. Sumimoto, Y. Yokoyama, I. Kuribayashi, Y. Mitsuuchi, T. Maeda, Y. Yamamoto, Y. Sagara and et al. (1990). "Structural and functional characterization of human aromatase P-450 gene." Eur J Biochem **193**(2): 559-65.

Toniolo, P. G., M. Levitz, A. Zeleniuch-Jacquotte, S. Banerjee, K. L. Koenig, R. E. Shore, P. Strax and B. S. Pasternack (1995). "A prospective study of endogenous estrogens and breast cancer in postmenopausal women." J Natl Cancer Inst **87**(3): 190-7.

Torres, M. (2003). "Mitogen-activated protein kinase pathways in redox signaling." Front Biosci **8**: d369-91.

Tsai, M. J. and B. W. O'Malley (1994). "Molecular mechanisms of action of steroid/thyroid receptor superfamily members." Annu Rev Biochem **63**: 451-86.

Tsuchiya, Y., M. Nakajima and T. Yokoi (2005). "Cytochrome P450-mediated metabolism of estrogens and its regulation in human." Cancer Lett **227**(2):

115-24.

Tsujimoto, Y. (1998). "Role of Bcl-2 family proteins in apoptosis: apoptosomes or mitochondria?" Genes Cells **3**(11): 697-707.

Vallejo, M., M. E. Gosse, W. Beckman and J. F. Habener (1995). "Impaired cyclic AMP-dependent phosphorylation renders CREB a repressor of C/EBP-induced transcription of the somatostatin gene in an insulinoma cell line." Mol Cell Biol **15**(1): 415-24.

van Delft, M. F. and D. C. Huang (2006). "How the Bcl-2 family of proteins interact to regulate apoptosis." Cell Res **16**(2): 203-13.

van Meeuwen, J. A., N. Korthagen, P. C. de Jong, A. H. Piersma and M. van den Berg (2007). "(Anti)estrogenic effects of phytochemicals on human primary mammary fibroblasts, MCF-7 cells and their co-culture." Toxicol Appl Pharmacol **221**(3): 372-83.

Wagner, J., L. Jiang and L. Lehmann (2008). "Phytoestrogens modulate the expression of 17alpha-estradiol metabolizing enzymes in cultured MCF-7 cells." Adv Exp Med Biol **617**: 625-32.

Walle, U. K. and T. Walle (2002). "Induction of human UDP-glucuronosyltransferase UGT1A1 by flavonoids-structural requirements." Drug Metab Dispos **30**(5): 564-9.

Wang, Y., K. W. Lee, F. L. Chan, S. Chen and L. K. Leung (2006). "The red wine polyphenol resveratrol displays bilevel inhibition on aromatase in breast cancer cells." Toxicol Sci **92**(1): 71-7.

Wang, Y., W. Man Gho, F. L. Chan, S. Chen and L. K. Leung (2008). "The red clover (Trifolium pratense) isoflavone biochanin A inhibits aromatase activity and expression." Br J Nutr **99**(2): 303-10.

Warner, M., B. Eskenazi, P. Mocarelli, P. M. Gerthoux, S. Samuels, L. Needham, D. Patterson and P. Brambilla (2002). "Serum dioxin concentrations and breast cancer risk in the Seveso Women's Health Study." Environ Health Perspect **110**(7): 625-8.

Watanabe, H., A. Suzuki, M. Goto, S. Ohsako, C. Tohyama, H. Handa and T. Iguchi

(2004). "Comparative uterine gene expression analysis after dioxin and estradiol administration." J Mol Endocrinol **33**(3): 763-71.

Wen, W., Z. Ren, X. O. Shu, Q. Cai, C. Ye, Y. T. Gao and W. Zheng (2007). "Expression of cytochrome P450 1B1 and catechol-O-methyltransferase in breast tissue and their associations with breast cancer risk." Cancer Epidemiol Biomarkers Prev **16**(5): 917-20.

Whitehead, S. A. and M. Lacey (2003). "Phytoestrogens inhibit aromatase but not 17beta-hydroxysteroid dehydrogenase (HSD) type 1 in human granulosa-luteal cells: evidence for FSH induction of 17beta-HSD." Hum Reprod **18**(3): 487-94.

Yamada-Mouri, N., S. Hirata and J. Kato (1996). "Existence and expression of the untranslated first exon of aromatase mRNA in the rat brain." J Steroid Biochem Mol Biol **58**(2): 163-6.

Yamamoto, S., E. Aizu, H. Jiang, T. Nakadate, I. Kiyoto, J. C. Wang and R. Kato (1991). "The potent anti-tumor-promoting agent isoliquiritigenin." Carcinogenesis **12**(2): 317-23.

Ye, L., W. M. Gho, F. L. Chan, S. Chen and L. K. Leung (2008). "Dietary administration of the licorice flavonoid isoliquiritigenin deters the growth of MCF-7 cells overexpressing aromatase." Int J Cancer.

Yoshidome, K., M. A. Shibata, C. Couldrey, K. S. Korach and J. E. Green (2000). "Estrogen promotes mammary tumor development in C3(1)/SV40 large T-antigen transgenic mice: paradoxical loss of estrogen receptoralpha expression during tumor progression." Cancer Res **60**(24): 6901-10.

Youle, R. J. and A. Strasser (2008). "The BCL-2 protein family: opposing activities that mediate cell death." Nat Rev Mol Cell Biol **9**(1): 47-59.

Yu, S. M. and S. C. Kuo (1995). "Vasorelaxant effect of isoliquiritigenin, a novel soluble guanylate cyclase activator, in rat aorta." Br J Pharmacol **114**(8): 1587-94.

Yu, Z., L. Zhang and D. Wu (2003). "[Genistein induced apoptosis in MCF-7 and T47D cells]." Wei Sheng Yan Jiu **32**(2): 125-7.

Yue, W. and A. Brodie (1993). "MCF-7 human breast carcinomas in nude mice as a

model for evaluating aromatase inhibitors." J Steroid Biochem Mol Biol **44**(4-6): 671-3.

Yue, W., S. J. Santner, S. Masamura, J. P. Wang, L. M. Demers, C. Hamilton and R. J. Santen (1998). "Determinants of tissue estradiol levels and biologic responsiveness in breast tumors." Breast Cancer Res Treat **49 Suppl 1**: S1-7; discussion S33-7.

Yue, W., D. Zhou, S. Chen and A. Brodie (1994). "A new nude mouse model for postmenopausal breast cancer using MCF-7 cells transfected with the human aromatase gene." Cancer Res **54**(19): 5092-5.

Yueh, M. F., N. Nguyen, M. Famourzadeh, C. P. Strassburg, Y. Oda, F. P. Guengerich and R. H. Tukey (2001). "The contribution of UDP-glucuronosyltransferase 1A9 on CYP1A2-mediated genotoxicity by aromatic and heterocyclic amines." Carcinogenesis **22**(6): 943-50.

Zhang, L., I. A. Khan, K. L. Willett and C. M. Foran (2003). "In vivo effects of black cohosh and genistein on estrogenic activity and lipid peroxidation in Japanese Medaka (Oryzias latipes)." J Herb Pharmacother **3**(3): 33-50.

Zhang, X., J. J. Jiao, B. R. Bhavnani and S. P. Tam (2001). "Regulation of human apolipoprotein A-I gene expression by equine estrogens." J Lipid Res **42**(11): 1789-800.

Zhao, H., Z. Tian, J. Hao and B. Chen (2005). "Extragonadal aromatization increases with time after ovariectomy in rats." Reprod Biol Endocrinol **3**: 6.

Zhou, C., D. Zhou, J. Esteban, J. Murai, P. K. Siiteri, S. Wilczynski and S. Chen (1996). "Aromatase gene expression and its exon I usage in human breast tumors. Detection of aromatase messenger RNA by reverse transcription-polymerase chain reaction." J Steroid Biochem Mol Biol **59**(2): 163-71.

Zhou, D. and S. Chen (1998). "Characterization of a silencer element in the human aromatase gene." Arch Biochem Biophys **353**(2): 213-20.

Zhou, D., P. Clarke, J. Wang and S. Chen (1996). "Identification of a promoter that controls aromatase expression in human breast cancer and adipose stromal

cells." J Biol Chem **271**(25): 15194-202.

Zhou, J., B. Gurates, S. Yang, S. Sebastian and S. E. Bulun (2001). "Malignant breast epithelial cells stimulate aromatase expression via promoter II in human adipose fibroblasts: an epithelial-stromal interaction in breast tumors mediated by CCAAT/enhancer binding protein beta." Cancer Res **61**(5): 2328-34.

Zhu, B. T. and A. H. Conney (1998). "Functional role of estrogen metabolism in target cells: review and perspectives." Carcinogenesis **19**(1): 1-27.

Zierau, O., S. Gester, P. Schwab, P. Metz, S. Kolba, M. Wulf and G. Vollmer (2002). "Estrogenic activity of the phytoestrogens naringenin, 6-(1,1-dimethylallyl)naringenin and 8-prenylnaringenin." Planta Med **68**(5): 449-51.

Zimarina, T. C., V. N. Kristensen, E. N. Imianitov and L. M. Bershtein (2004). "[Polymorphisms of CYP1B1 and COMT in breast and endometrial cancer]." Mol Biol (Mosk) **38**(3): 386-93.

# ACKNOWLEGEMENTS

I would like to express my sincere gratitude to my supervisor, Dr. L.K. Leung, for his great support, encouragement and patient guidance during the whole period of my study. I benefit a lot from his guidance and advice on my PhD project. In addition, I would like to thank Dr. T.T. Kwok and his students for their great help on my project. I would like to thank Dr. F.L. Chan for his useful suggestion on how to establish the animal model. Also, I would like to thank my external examiner Dr Jennifer M.F.Wan.

I am grateful to all my labmates working in MMW507, for their friendship, assistance and technical support. Moreover, I would like to thank all my colleagues and friends in the Department of Biochemistry, The Chinese University of Hong Kong, for their kind assistance.

Finally, special thanks are given to my parents, my brother and my boyfriend for their love and encouragement.

# ABSTRACT

Breast cancer is one of the most prevalent female cancers in Hong Kong and western countries. Prolonged exposure to estrogen has been associated with increased risk of breast cancer. Many enzymes are responsible for estrogen metabolism, for instance, aromatase (CYP19) is responsible for biosynthesis; CYP1 family enzymes hydroxylate estrogen; COMT (catechol-O-methyltransferase) inactivates the hydroxyestrogen; and UDP-glucuronosyltransferase 1A1 (UGT1A1) eliminates the estrogen metabolites. In this project, we employed cell and animal models to address estrogen metabolism-related questions under the influence of endocrine disruptors.

TCDD is a prototype compound of a whole class of halogenated aromatic hydrocarbons termed dioxin-like contaminants, which are also known to be endocrine disruptors. Because of their persistence in the environment dioxins are one of the most concerned classes of carcinogens. Humans can be exposed to this pollutant through contaminated food, air, drinking water, etc. We found that pre-ovariectomy administration of TCDD could significantly reduce aromatase expression in the brain but increase the expression in the adipose tissue. Our results suggested that the timing of exposure to the toxicant could determine the estrogenicity of TCDD.

Because of the structural resemblance to the female hormone, phytoestrogen is another important class of endocrine disruptor. In the present project, we evaluated the effects of phytoestrogens isoliquiritigenin (ILN), hesperetin (HES), genistein,

(GEN) and naringenin (NAR) on estrogen metabolism and also their effects on MCF-7 tumor growth in ovariectomized nude mice. We found that these phytoestrogens had differential effect on MCF-7 xenografts. NAR and GEN had totally different responses in the tumor growth. In contrast, ILN and HES only deterred MCF-7 xenograft growth when CYP19 was overexpressed in the graft.

The present project indicated that endocrine disruptors can alter the metabolism of estrogen; however, the significance of this alteration may be specific to tissues' phenotype and the timing of exposure.

# 摘要

在香港和西方國家裏，乳腺癌是女性常見的腫瘤。長期接觸雌激素和乳腺癌的發病率上升有著緊密的聯系。

衆多的酶參與了雌激素的代謝，芳香化酶(CYP19)是雌激素生物合成過程中的關鍵酶；CYP1 家族酶羥基化雌激素；兒茶酚氧位甲基轉移酶(COMT)甲基化羥基化的雌激素；尿苷二磷酸葡萄糖醛酸酶(UGT1A1) 進一步消除雌激素的代謝産物。在此項研究中，我們通過用細胞和動物的模型來闡述在內分泌幹擾素作用條件下雌激素代謝的有關問題。

鹵化芳香化氫化物被廣泛認為是一種內分泌幹擾素，而二惡英是鹵化芳香化氫化物的一個常見化合物。因為它們在環境中長期留存不易分解，二惡英被認為是一類重要的致癌物。二惡英汙染物主要是通過食用被汙染的食物，空氣以及飲用水等途徑。研究發現，在切卵巢前灌胃二惡英的大鼠中，芳香化酶的表達在腦中降低，相反芳香化酶的表達在脂肪組織中增加。結果表明，二惡英能否發揮其類雌激素的特性和二惡英的作用時間有關。

植物雌激素和雌激素的結構類似，屬於另一類內分泌幹擾素。在此項研究中，我們調查了異甘草素(ILN)，橙皮素(HES)，染料木素(GEN)和柚皮素(NAR)對雌激素代謝的影響，並進一步研究了它們在小鼠中對乳腺癌的作用。研究發現，這些不同的植物雌激素對 MCF-7 腫瘤有著不同的影響。NAR 和 GEN 對腫瘤有著完全相反的作用。相反 ILN 和 HES 抑制乳腺癌細胞 MCF-7aro 的增殖。

研究表明，內分泌幹擾素能夠影響雌激素的代謝，然而它們能否特異性發揮作用與其作用的組織及其時間相關。

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AhR | Aryl Hydrocarbon Receptor |
| AP-1 | Activator protein-1 |
| Amp | Ampicilin |
| Bcl-2 | B-cell leukemia/lymphoma 2 |
| bFGF | Basic fibroblast growth factor |
| bp | Base pair |
| BSA | Bovine serum albumin |
| cAMP | Cyclic adenosine monophosphate |
| cDNA | Complementary deoxyribonucleic Acid |
| C/EBP | CCAAT/enhancer binding protein |
| COMT | Catechol-O-methyltransferase |
| COX-2 | Cyclooxygenase-2 |
| CRE | cAMP response element |
| CREB | CRE binding protein |
| CYP | Cytochrome P450 |
| DAG | Diacylglycerol |
| dATP | Deoxyadenosyl triphosphate |
| dCTP | Deoxycytosinyl triphosphate |
| dGTP | Deoxyguanosinyl triphosphate |
| DMEM/F12 | Dulbecco's Modification of Eagle's Medium/Ham's F12 |
| DMSO | Dimethyl sulfoside |
| DNA | Deoxynucleic acid |
| dNTP | Deoxyribonucleotide Triphosphate |
| DTT | Dithiothreitol |
| dTTP | Deoxythymidunyl triphosphate |
| E2 | 17β-estradiol |
| E.coli | Escherichea coli |
| EDTA | Ethylenediaminetetraacetate |
| EGF | Epidermal growth factor |
| EGFR | Epigermal growth factor receptor |
| ERBB2 | erythroblastic leukemia viral oncogene homolog 2 |
| ERE | Estrogen response element |
| ERK 1/2 | Extracellular signal-regulated kinase 1/2 |
| ER | Estrogen receptors |
| EtOH | Ethanol |
| FBS | Fetal bovine serum |
| Fos | v-fos FBJ murine osteosarcoma viral oncogene homolog |
| FW | Formula weight |
| GEN | Genistein |
| HEPES | N-2-hydroxy-ethyl-piperazine-N'-2- |

| | |
|---|---|
| | Ethane-sulfonic acid |
| HES | Hesperetin |
| HER2 | Human epidermal growth factor receptor 2 |
| IL | Interleukin |
| ILN | Isoliquiritigenin |
| $IP_3$ | Iinositol-1,4,5-triphosphate |
| JNK | c-Jun N-terminal kinase |
| Jun | v-jun avian sarcoma virus 17 oncogene homolog |
| kb | Kilo-base |
| kDa | Kilo-dalton |
| LB | Luria-Broth Medium |
| LBD | Ligand binding domain |
| LPS | lipopolysaccharide |
| MAP | Mitogen-activated protein |
| MAPK | Mitogen-activated protein kinase |
| MBD | Membrane binding domain |
| MEK | Mitogen-activated protein / Extracellular signal-regulated kinase kinase |
| mRNA | Messenger ribonucleic acid |
| MTT | 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide |
| NAR | Naringenin |
| NFAT | Nuclear factor of activated T-cells |
| NFκB | Nuclear factor kappa B |
| NF-IL6 | Nuclear factor interleukin-6 |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| $PIP_2$ | phosphoinositol-4,5-bisphoaphate |
| PI3 kinase | phosphatidylinositol 3-kinase |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| RNA | Ribonucleic acid |
| RNase | Ribonuclease |
| rpm | Revolutions per minute |
| RPMI 1640 Medium | Rosewell Park Memorial Institute Tissue Culture Medium 1640 |
| RT-PCR | Reverse transcription-polymerase chain reaction |
| SD | Female Sprague Dawley |
| SDS | Sodium dodecyl sulfate |
| TAE | Tris-acetate-EDTA |
| TCDD | 2,3,7,8-tetrachlorodibenzo-para-dioxin |
| TGFα | Transforming growth factor alpha |
| TGFβ-1 | Transforming growth factor beta-1 |
| Tris | Trizma base |

| | |
|---|---|
| UGT1A1 | UDP-glucuronosyltransferase 1A1 |
| XRE | Xenobiotic Response Element |

# TABLE OF CONTENTS



## Table of Contents