Modulation of Estrogen Oxidative Metabolism by Botanicals (Hops and Licorice) Used for Women's Health

BY

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THESIS

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This thesis is dedicated to my wife Xiaoyu Hu, my parents Yaohua Sun and Jinfei Wang.

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CONTRIBUTION OF AUTHORS

Figure 1 was generated by Dr. Tareisha Dunlap and modified by me in this thesis. The information in Chapter 3 and 4 was reprinted in part with format changes from the published paper, “Dunlap TL, Wang S, Simmler C, Chen SN, Pauli GF, Dietz BM, and Bolton JL. “Differential effects of glycyrrhiza species on genotoxic estrogen metabolism: licochalcone A downregulates P450 1B1, whereas isoliquiritigenin stimulates it” Chemical Research in Toxicology 28(8), 1584-1594 (2015)”, in which I was a primary author. Dr. Charlotte Simmler generated Table I and Figure 8, and Dr. Tareisha Dunlap generated Figure 19, 20, 22, 23C, and 24, for the original publication and the figures were reformatted in this thesis. The data in Table II was generated by Lingyi Huang. The information in Chapter 5 was reprinted in part with format changes from the published paper, “Wang S, Dunlap TL, Howell CE, Mbachu OC, Rue EA, Chen SN, Pauli GF, Dietz BM, and Bolton JL. “Hop (Humulus Lupulus L.) extract and 6-prenylnaringenin induce P450 1A1 catalyzed estrogen 2-hydroxylation” Chemical Research in Toxicology (Epub 2016)”, in which was a primary author. Dr. Tareisha Dunlap generated Figure 32, 34A-C, and 39, for the original publication and the figures were reformatted in this thesis. Huali Dong generated data and I graphed for Figure 28. All the other figures and tables were generated by me and the reprints for Chapter 3 and 4 were made with permission from the journal *Chemical Research in Toxicology*.

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LIST OF ABBREVIATIONS

AhR	Aryl hydrocarbon receptor
AI	Aromatase inhibitor
ARNT	Aryl hydrocarbon receptor nuclear translocator
COMT	Catechol-O-methyl transferase
CYP	Cytochrome P450 enzyme
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E_1	Estrone
E_2	17β-Estradiol
ER	Estrogen receptor
EROD	Ethoxyresorufin O deethylase
ESI	Electrospray ionization
FBS	Fetal Bovine Serum
FDA	Food and drug administration
GG	*Glycyrrhiza glabra*
GI	*Glycyrrhiza inflata*
GU	*Glycyrrhiza uralensis*
GSH	Glutathione
GST	Glutathione S transferase
HER2	Human epidermal growth factor receptor 2
HPLC	High-performance liquid chromatography
HRT	Hormone replacement therapy
IX	Isoxanthohumol
LicA	Licochalcone A
LigC	Isoliquiritigenin

LIST OF ABBREVIATIONS (continued)

LigF	Liquiritigenin
$MeOE_1$	Methoxyestrone
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor kappa B
NQO1	NAD(P)H quinone oxidoreductase 1
PAH	Polycyclic aromatic hydrocarbons
PARP	Poly ADP ribose polymerase
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
6-PN	6-Prenylnaringenin
8-PN	8-Prenylnaringenin
ROS	Reactive oxygen species
RNA	Ribonucleic acid
SERM	Selective estrogen receptor modulator
SULT	Sulfotransferase
TCDD	2,3,7,8-Tetrachlorodibenzodioxin
TMS	2,3',4,5'-Tetramethoxystilbene
UGT	Uridine 5'-diphospho-glucuronosyltransferase
WHI	Women’s Health Initiative
XH	Xanthohumol
XRE	Xenobiotic response element

SUMMARY

Breast cancer is the most commonly diagnosed cancer in women in the United States, representing about one third of all new cancer cases, and is also the second leading cause of cancer related deaths in women. Although there are various risk factors for breast cancer, increased exposure to the endogenous hormone, estrogen, significantly increases the risk of breast cancer. There are two major mechanisms of estrogen carcinogenesis; the hormonal pathway and the chemical pathway. The hormonal pathway involves estrogen binding to the estrogen receptor, which leads to the activation of estrogen receptor signaling. The signaling cascade increases cell proliferation and chances of sporadic mutations, which increases the chance of cancer initiation and promotion. The chemical estrogen carcinogenesis pathway involves P450 catalyzed oxidation of estrogens ultimately forming genotoxic metabolites.

In breast tissues, the chemical estrogen carcinogenesis pathway involves hydroxylation on the 2- and 4- positions of estrogens catalyzed by P450 1A1 and P450 1B1, respectively. These P450s are major extrahepatic enzymes expressed in the mammary gland. The 4-hydroxylated estrogen catechols can be oxidized to reactive estrogen-3,4-quinone electrophiles that are known to alkylate DNA and form depurinating adducts. The oxidation from catechol to quinone also increases reactive oxygen species (ROS) production, which leads to increased DNA oxidation. The reactive estrogen-3,4-quinones and ROS can induce cancer initiation and promotion due to their genotoxic properties. On the other hand, 2-hydroxylated estrogen catechols represent a detoxification pathway, because 2-methoxyestradiol has been shown to be an anti-angiogenesis and anti-proliferative agent. As a result, the 4-hydroxylation

pathway catalyzed by P450 1B1 represents the genotoxic pathway and the 2-hydroxylation catalyzed by P450 1A1 represents the detoxification pathway. Therefore, agents that can inhibit the 4-hydroxylation pathway or induce the 2-hydroxylation pathway could have a preventive effect on breast cancer.

Botanical dietary supplements have been widely used in the United States and also frequently used by women as an alternative to menopausal hormone therapy. As women are exposed to these botanicals with reported anti-inflammatory and chemopreventive activities, we hypothesized that they could have other health benefits including their ability to modulate estrogen chemical carcinogenic pathways. In this study, several botanical extracts were tested for their effects on estrogen 2- and 4-hydroxylation pathways in breast cells and in the ACI rat model.

A LC-MS/MS method was optimized to quantify 2- and 4-methoxyestrone as markers for estrogen 2- and 4-hydroxylation activity. Non-tumorigenic, estrogen receptor (ER) negative, breast epithelial MCF-10A cells were first used to study estrogen oxidative metabolism *in vitro*. Three different licorice species [*Glycyrrihiza uralensis (GU), G. glabra, and G. inflate (GI)*] were tested which had differential effects on estrogen oxidative metabolism with GI down-regulating estrogen 2- and 4-hydroxylation metabolism and GG and GU slightly inducing these hydroxylation pathways. Some major bioactive compounds from licorice including isoliquiritigenin (LigC), liquiritigenin (LigF), and the GI unique compound licochalcone A (LicA), were also tested. LicA was found to be an antagonist of the up-stream aryl hydrocarbon

receptor (AhR). LicA also showed dose-responsive down-regulation of estrogen 2- and 4-hydroxylation metabolism, while LigC increased the metabolism. *In vivo* effects of LicA on estrogen oxidative metabolism was also measured in the ACI rat model. LicA significantly down-regulated the estrogen oxidative metabolism in this animal model consistent with the *in vitro* results.

The strobili of hops (*Humulus lupulus*) extract were also analyzed for their influence on oxidative estrogen metabolism. The LC-MS/MS results revealed the preferential up-regulation of the 2-hydroxylation detoxification pathway in two different breast cell lines by hops. In non-tumorigenic ER negative MCF-10A cells, hops slightly induced the 2-hydroxylation pathway, with little effect on the 4-hydroxylation pathway. In tumorigenic ER positive MCF-7 cells, hops significantly induced both estrogen 2-hydroxylation and 4-hydroxylation. Hop's major bioactive prenylated compounds 6-prenylnaringenin (6-PN), 8-prenylnaringenin (8-PN), isoxanthohumol (IX), and xanthohumol (XH) were also tested in these cell lines. 6-PN was found to correlate with the induction activity of the hops extract. 6-PN preferentially up-regulated estrogen 2-hydroxylation over 4-hydroxylation metabolism through preferential induction of P450 1A1 over P450 1B1 in both cell lines. 6-PN was also identified as an AhR agonist through induction of xenobiotic response element activation. 8-PN, IX, and XH did not have significant effects on the AhR in MCF-7 cells.

In conclusion, this study demonstrates that GI and hops could potentially reduce the risk of breast cancer through estrogen oxidative metabolism modulation. Thus,

standardization of the botanical dietary supplements to the key bioactive compounds, can provide women with botanical supplements containing a better safety profile, reproducibility of biological outcomes, as well as beneficial effects on breast cancer prevention. Future studies are needed to test the botanicals *in vivo* and balance the multiple biological effects of the phytoconstituents in women's health to obtain safe and healthy botanical dietary supplements.

1. INTRODUCTION

1.1. Breast Cancer

Breast cancer is the most commonly diagnosed cancer in women in the United States (Siegel et al., 2016). Epidemiology studies indicate that one in eight women will develop breast cancer during her life time. In 2016, the American Cancer Society estimates 246, 660 breast cancer diagnosis and 40, 450 estimated deaths due to breast cancer (Siegel et al., 2016). Since its prevalence and mortality rates are high, breast cancer is one of the most well studied cancers. Research publications on breast cancer have dramatically increased and eight new breast cancer drugs have been approved by FDA in the past ten years (Gupta et al., 2016). Although the rate of breast cancer diagnosis has been relatively unchanged in the past decade, the breast cancer mortality rate has decreased possibly due to enhanced early detection methods and new treatment options (DeSantis et al., 2014; Miller et al., 2016; Siegel et al., 2012).

Breast cancer can be classified into several subtypes based on disease state, area of origin, and receptor status. There are three major receptors involved in breast cancer classification: estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). These classifications helped drug development target different subtypes of breast cancer based on the receptor status. Treatments include aromatase inhibitors (AI, i.e. letrozole) and selective estrogen receptor modulators (SERM, i.e. tamoxifen) for ER+ breast cancers, HER2 antibody trastuzumab for HER2+ breast cancers, and chemotherapy agents, such as taxol, for triple negative breast cancer (Goldhirsch et al., 2011). Breast cancer can also be classified into two major groups, ductal and lobular carcinoma, which is based on the area of origin of the cancer. Ductal

carcinoma is the most common type of breast cancer including both in situ and invasive types. The lobular carcinoma is mostly invasive and harder to detect by a mammogram (Stenkvist et al., 1983). Breast cancer are also frequently classified based on the size of the tumor (T), whether it is spread to lymph node (N), and whether it has metastasized (M) to different stages of breast cancer, which indicates the cancer severity.

Various factors can contribute to increased breast cancer risk including age, race, living style, genetic factors, diet, and lack of physical exercises (MacMahon et al., 1970; Pharoah et al., 1997) (Carmichael and Bates, 2004; Willett, 2001). Just like any other cancer, breast cancer is an age related disease that is mostly diagnosed in women after 50 years of age. The accumulation of genetic mutations due to various factors is believed to eventually lead to breast cancer initiation, which are difficult to target. Genetic mutation of breast cancer susceptibility genes, such as *BRCA*-1 or 2, which are expressed in breast cells and responsible for DNA repair, has been found to be associated with increased risk of breast cancer (Rahman and Stratton, 1998).

1.2. Estrogen and Breast Cancer

Prolonged exposure to estrogens is a known risk factor for breast cancer (Yager and Davidson, 2006). More than a hundred years ago, the British physician George Beatson showed for the first time that removal of ovaries from women with breast cancer reduced the tumor and disease burden. This demonstrated estrogen dependency of breast cancer and by limiting the endogenous hormone, estrogen-dependent breast cancer malignancies could be dramatically altered (Beatson, 1983). The link between hormone replacement therapy and estrogen carcinogenesis has been demonstrated from the results

of the Women's Health Initiative in 2002 (Rossouw et al., 2002). More than 16,000 healthy postmenopausal women were recruited from 1993 to 1998 to study the effect of Prempro (Premarin, medroxyprogesterone acetate) on postmenopausal women's health. However, the study was halted early due to adverse effects including significant increased breast cancer risk (27%), coronary heart disease (29%), and stroke (41%). More strikingly, a follow-up study on these women in a 15 year span indicated a sustained higher risk of breast cancer in the Prempro group vs the Placebo group, with a cumulative higher risk of 55% (Chlebowski et al., 2013). Thus, the risk of breast cancer increases with enhanced exposure to estrogen, which often results from hormone therapy, late menopause, and early puberty (Colditz, 1998; Feigelson and Henderson, 1996; Feuer et al., 1993; MacMahon et al., 1970; Wolff et al., 1996).

There are two commonly accepted mechanisms of estrogen carcinogenesis. The hormonal pathway involves estrogen binding to estrogen receptor (ER), activating ER mediated signaling cascade, which increases cell proliferation and chances of genetic mutations. The other pathway, often referred to as the chemical pathway, is associated with the 4-hydroxylation pathway of estrogen (Figure 1) (Feigelson and Henderson, 1996; Henderson and Feigelson, 2000; Shang, 2006; Yager and Davidson, 2006).

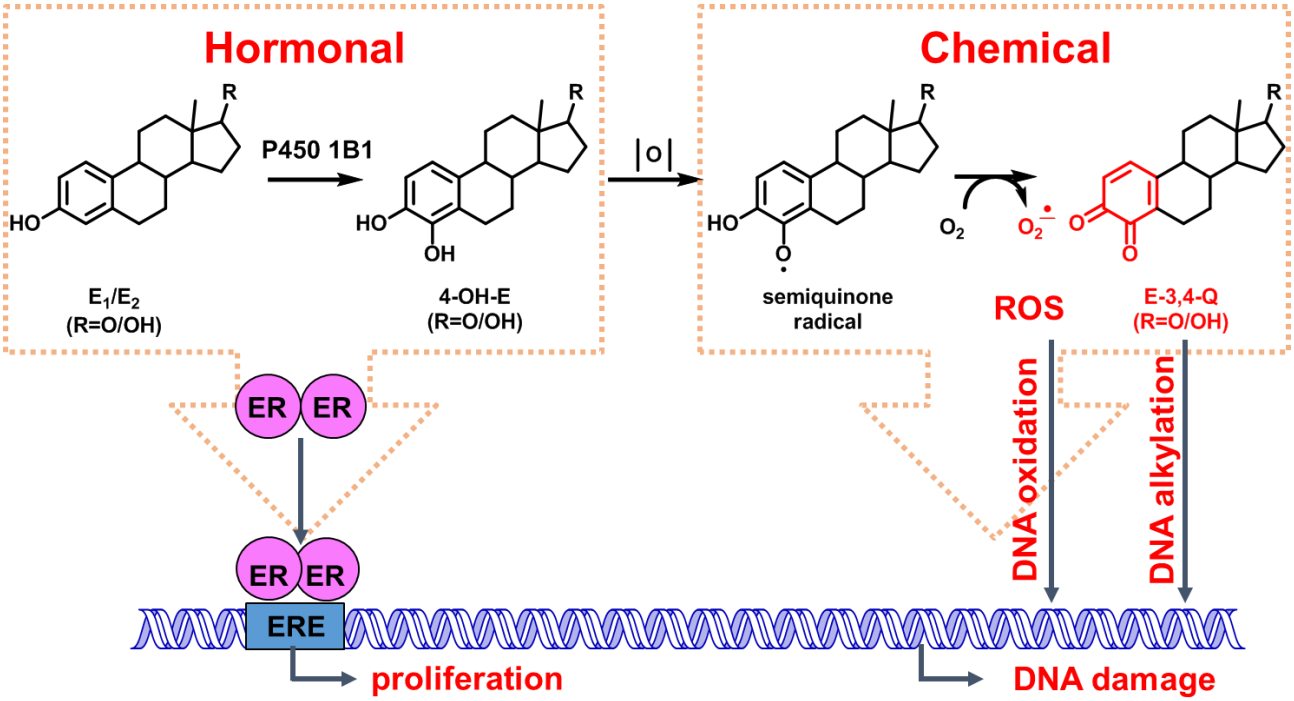


Figure 1. Scheme of estrogen hormonal and chemical carcinogenesis pathways. Estrogen hormonal carcinogenesis pathway is mediated via estrogens binding to estrogen receptor (ER) and leading to ER signaling and cell proliferation. Estrogen chemical carcinogenesis is mediated via the production of reactive estrogen quinone electrophile (E-3,4-Q) and reactive oxygen species (ROS), which causes DNA damage through DNA alkylation and oxidation. E_1, estrone; E_2, estradiol. (Figure modified from a figure generated by Dr. Tareisha Dunlap)

1.3. Estrogen Hormonal Carcinogenesis

Upon binding of a ligand, ERα goes through conformational changes, leading to dissociation of chaperone proteins and dimerization with another ER protein. The ligand bound ER dimer translocates into the nucleus and binds to the estrogen response element (ERE) (Figure 2). This represents the classical genomic pathway for ER activation. After the recruitment of co-activators to the ERE promoter region, the receptor DNA complex initiates ER targeted gene transcription (Metivier et al., 2003). ER signaling leads to the expression of various target genes that promote cell proliferation, facilitating gene

mutations, which eventually might lead to the initiation of cancer (Clemons and Goss, 2001; Williamson and Lees-Miller, 2011). Estrogen receptor induced cell proliferation can also contribute to tumor promotion from the initiated tumor cells. Additionally, *in vitro* studies have revealed that the inflammatory pathway mediated by NF-κB acts synergistically with ER on promoting cancer cell growth and survival (Frasor et al., 2009). Clinical evidence and animal studies supported the important role of NF-κB activation in high risk subsets of ER+ breast cancers. Inhibition of NF-κB activation was shown to restore the sensitivity of advanced ER+ breast cancer to traditional therapies (Zhou et al., 2005).

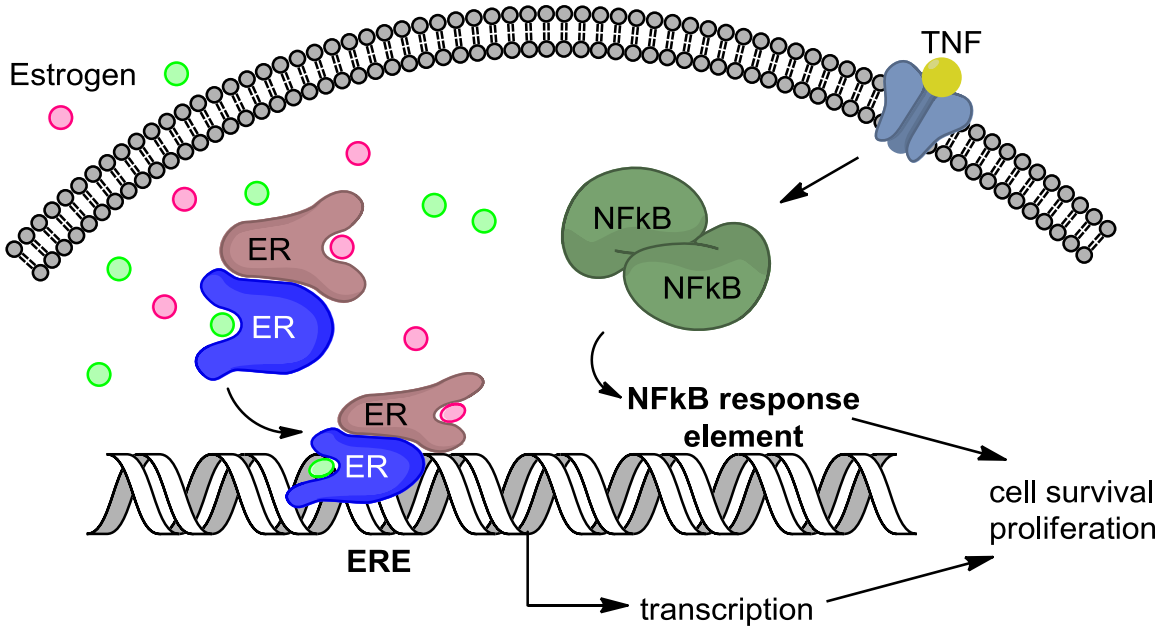


Figure 2. ER and NFκB signaling synergistically promote breast carcinogenesis. Estrogen receptor signaling pathway activated by estrogens (mainly estradiol and estrone) and NFκB signaling pathway activated by inflammatory cytokines both contribute to cell survival and proliferation. These two pathways could work synergistically to promote breast cancer.

1.4. Estrogen Chemical Carcinogenesis

1.4.1. Estrogen phase I and phase II metabolism

Estrogens are mainly produced in the ovaries for pre-menopausal women and in adipose tissues and adrenal gland for postmenopausal women (Siiteri, 1982). Estrogens, mainly estradiol (E_2) and estrone (E_1), are converted from testosterone and androstenedione by aromatase (P450 19A1) (Figure 3). E_2 and E_1 are inter-convertible through the enzyme 17β-hydroxysteroid dehydrogenase (17β-HSD).

Estrogen 2-hydroxylation and 4-hydroxylation are the major phase I oxidative metabolism of estrogen, which are catalyzed by P450 1A1 and P450 1B1, respectively, in the mammary tissue (Figure 3) (Spink et al., 1998). In liver, P450 1A2 and 3A4 are mainly responsible for estrogen 2-hydroxylation (80%) and 4-hydroxylation (20%) (Tsuchiya et al., 2005).

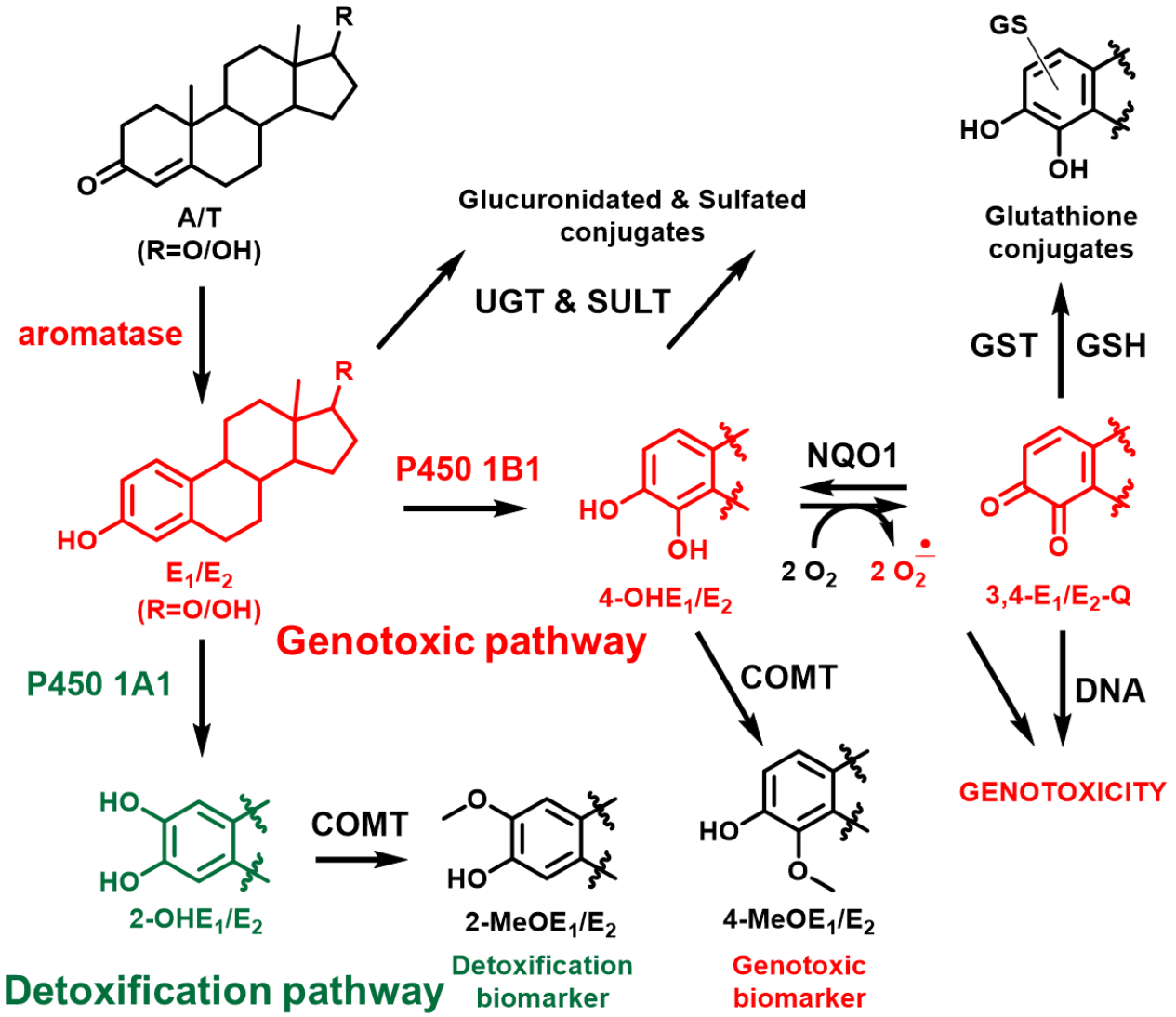


Figure 3. Scheme of estrogen production and metabolism pathways. Estrogens (estrone and estradiol) are converted from androstenedione and testosterone by the enzyme aromatase. Estrogens can be metabolized by P450 1A1 and P450 1B1 to 2-hydroxylated and 4-hydroxylated estrogen catechols. 4-Hydroxy estrogens can be oxidized to form 3,4-estrogen quinones and produce reactive oxygen species, which lead to genotoxicity. Estrogen quinones can be reduced by NQO1 to catechols or conjugated to GSH catalyzed by GST. The estrogens and metabolites can also be glucuronidated and sulfated by UGT and SULT to form conjugates. The estrogen catechols can be methylated by COMT to form more stable 2- and 4-methoxylatd estrogens, which can be used as biomarkers for the 2- and 4-hydroxylation pathway. (GSH, glutathione; GST, glutathione S transferase; UGT, UDP-Glucuronosyltransferase; SULT, Sulfotransferase)

P450 1A1 was first discovered as the aryl hydrocarbon hydroxylase thanks to its activity in catalyzing the hydroxylation of various polycyclic aromatic hydrocarbons (PAH) (Beresford, 1993). P450 1A1 bioactivates the conversion of PAHs such as benzo[*a*]pyrene to the final carcinogenic metabolite benzo[*a*]pyrene diol epoxide (Uno et al., 2004). In estrogen metabolism, P450 1A1 mainly catalyzes the 2-hydroxylation pathway to form 2-hydroxylated estrogen catechols.

P450 1B1 was identified in 1994, long after the discovery of P450 1A enzymes (Bhattacharyya et al., 1994; Sutter et al., 1994). Although being grouped in to the same subfamily, P450 1B1 shares only 37% homology with P450 1A1 and displays distinct catalytic activity towards various substrates, including estrogen 4-hydroxylation over 2-hydroxylation activity. However, these two enzymes in breast tissues can both be regulated through aryl hydrocarbon receptor (AhR) and are thus included in the P450 1 family (Yanagiba et al., 2009; Yang et al., 2008b). Research has revealed the role of P450 1B1 in the pathogenesis of various diseases (Sutter et al., 1994; Tang et al., 1996). In particular, it catalyzes the metabolism of several polycyclic aromatic hydrocarbon compounds to form reactive metabolites, such as benzo[*a*]pyrene diol epoxide (Lee et al., 2003; Shimada et al., 1996). *In vitro* determinations have indicated higher levels of P450 1B1 expression in breast cancer cells versus normal breast cells (McFadyen et al., 1999; Spink et al., 1998).

The mechanism of estrogen chemical carcinogenesis is associated with estrogen oxidative metabolism, more specifically, the 4-hydroxylation of estrogen metabolism (Yager, 2015). The 2- and 4-hydroxylated estrogen catechols formed can be readily oxidized to reactive quinone electrophiles and produce reactive oxidative species (ROS).

The estrogen quinone electrophiles react with DNA and protein to form DNA/protein adducts. Depurinating DNA adducts are formed under physiological conditions by these reactive quinones, including 4-OHE-1-N7-Guanine, 4-OHE-1-N3-Adenine, and to a much lesser extent, 2-OHE-6-N3-Adenine, which could lead to genotoxicity (Zahid et al., 2006; Zhao et al., 2006). Depurinating adducts from E-3,4-quinone have also been detected in breast epithelial cells *in vitro* as well as in animal studies (Chen et al., 2004; Li et al., 2004; Zahid et al., 2006). Additionally, the formation of E-3,4-quinone leads to elevated ROS levels thus contributing to genotoxicity and cellular damage due to oxidative damage of DNA, lipids, and proteins (Yager, 2015). Studies have shown that treatment of E2 in breast cell lines caused rapid production of ROS and elevated levels of 8-oxo-2'-deoxyguanosine (8-oxo-dG), which is produced by guanine oxidation and has also been frequently used as a biomarker to measure DNA oxidation (Felty et al., 2005; Haghdoost et al., 2005).

Phase II conjugation metabolism also plays a vital role in estrogen metabolism. Major metabolites (>90%) following oral estradiol administration have been shown to be estrone, estrone sulfate and glucuronide conjugates (Stanczyk et al., 2013). The conjugative metabolism can be viewed as a detoxification pathway as it transforms estrogens to non-reactive hydrophilic forms which increases the rate of excretion (Tukey and Strassburg, 2000). Glucuronidation and sulfation are the major phase II metabolic pathways of estrogens catalyzed by uridine 5'-diphospho-glucuronosyltransferases (UGT) and sulfotransferases (SULT), respectively (Stanczyk et al., 2013). Although phase II conjugation takes place mainly in the liver, extrahepatic conjugation does occur with the isoforms UGT1A1 and SULT1E1 being most active in estrogen conjugative metabolism (Desai et al., 2003; Negishi et al., 2001) (Figure 3). Other than glucuronidation and

sulfation, methylation of estrogen catechols catalyzed by catechol-O-methyl transferase (COMT), reduction of estrogen quinones to catechols by NAD(P)H–quinone oxidoreductase 1 (NQO1), as well as conjugation of estrogen quinones to glutathione catalyzed by glutathione S-transferases (GST) are the other major phase II metabolism enzymes (Butterworth et al., 1997; Raftogianis et al., 2000). NQO1 is frequently regarded as a chemopreventive enzyme and its expression can be induced through the activation of the Nrf2/Keap1 pathway (Talalay, 2000; Wakabayashi et al., 2004). Consequently, Nrf2/Keap1 activation and NQO1 induction have been used as an indicator to determine the chemopreventive activity of compounds (Kwak et al., 2004a; Kwak et al., 2004b).

1.4.2. P450 1A1/1B1 enzyme regulation

Regulation of P450 1A1 and 1B1 is mediated through the nuclear receptor AhR (Figure 4). Upon binding with an AhR ligand, the aryl hydrocarbon receptor undergoes conformational changes and dissociates from its chaperone proteins such as HSP90, translocate into the nucleus, forms a dimer structure with aryl hydrocarbon nuclear translocator (ARNT), and binds the xenobiotic response element (XRE). AhR regulates a variety of phase I and II enzymes and P450 1A1 and 1B1 are the two major enzymes in its gene battery (Tijet et al., 2006).

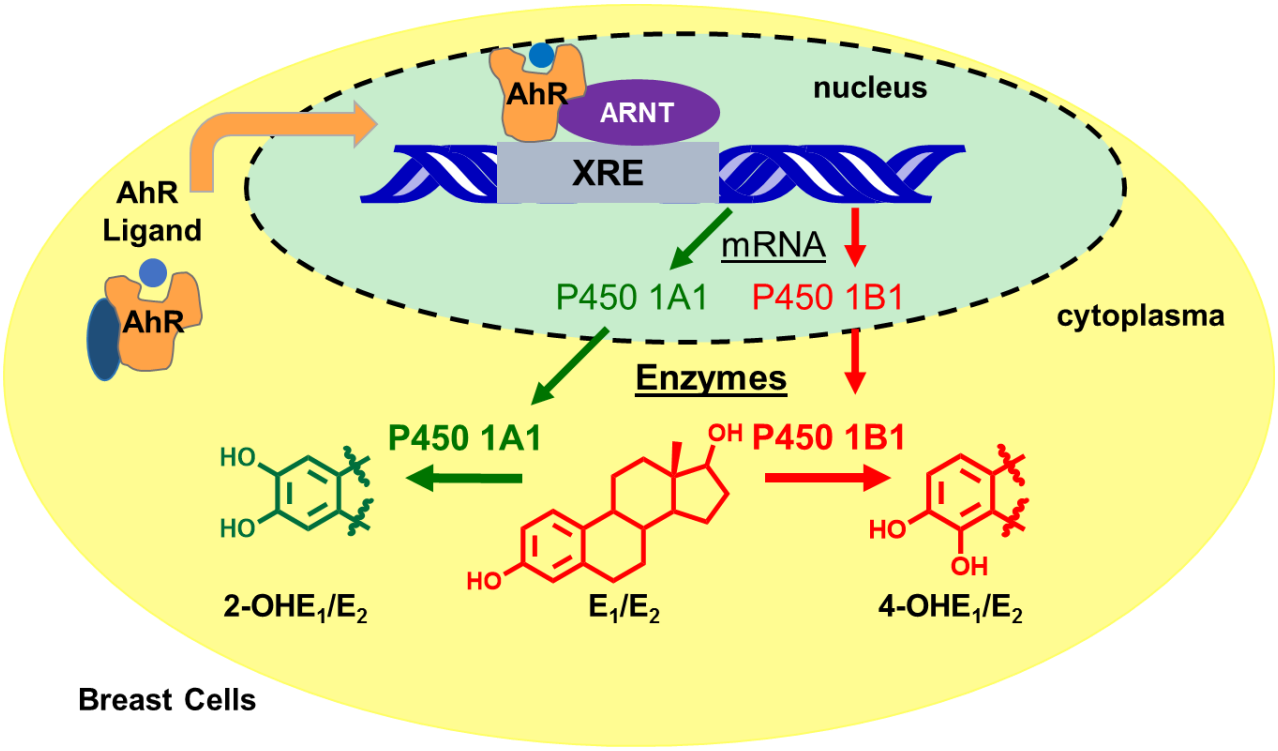


Figure 4. Aryl hydrocarbon receptor (AhR) signaling in breast cells. Nuclear receptor AhR undergoes conformational changes and disassociates with its chaperone protein upon binding with an AhR ligand. The ligand bound AhR translocates into the nucleus and forms heterodimer with the aryl hydrocarbon receptor nucleus translocator (ARNT). The heterodimer further binds to the promoter region of the xenobiotic response element (XRE) and lead to the transcription of target genes. P450 1A1 and 1B1 are the major proteins regulated by AhR in breast cells, which are responsible for the 2- and 4-hydroxylation of estrogens, respectively.

In addition to the AhR pathway, several groups have shown that P450 1B1 can also be up-regulated via the inflammatory pathway, while P450 1A1 was either not affected or downregulated (Han et al., 2010; Kamel et al., 2012; Patel et al., 2014; Šmerdová et al., 2014) (Figure 5). P450 1B1 was shown to be significantly induced via cytokine treatment in breast, endometrium, and liver cells, and was proposed to be regulated via p38 phosphorylation pathway that specifically targeted P450 1B1. Studies have also shown the

suppressive effect of ER signaling pathways on the regulation of P450 1A1 and to a much lesser extent, P450 1B1 (Angus et al., 1999; Spink et al., 1998; Tsuchiya et al., 2004). These new findings have indicated the complexity of estrogen chemical carcinogenesis, but also provided new evidence on the molecular mechanisms of differential regulation of P450 1A1 and 1B1 and thus provide different strategies for prevention of estrogen chemical carcinogenesis.

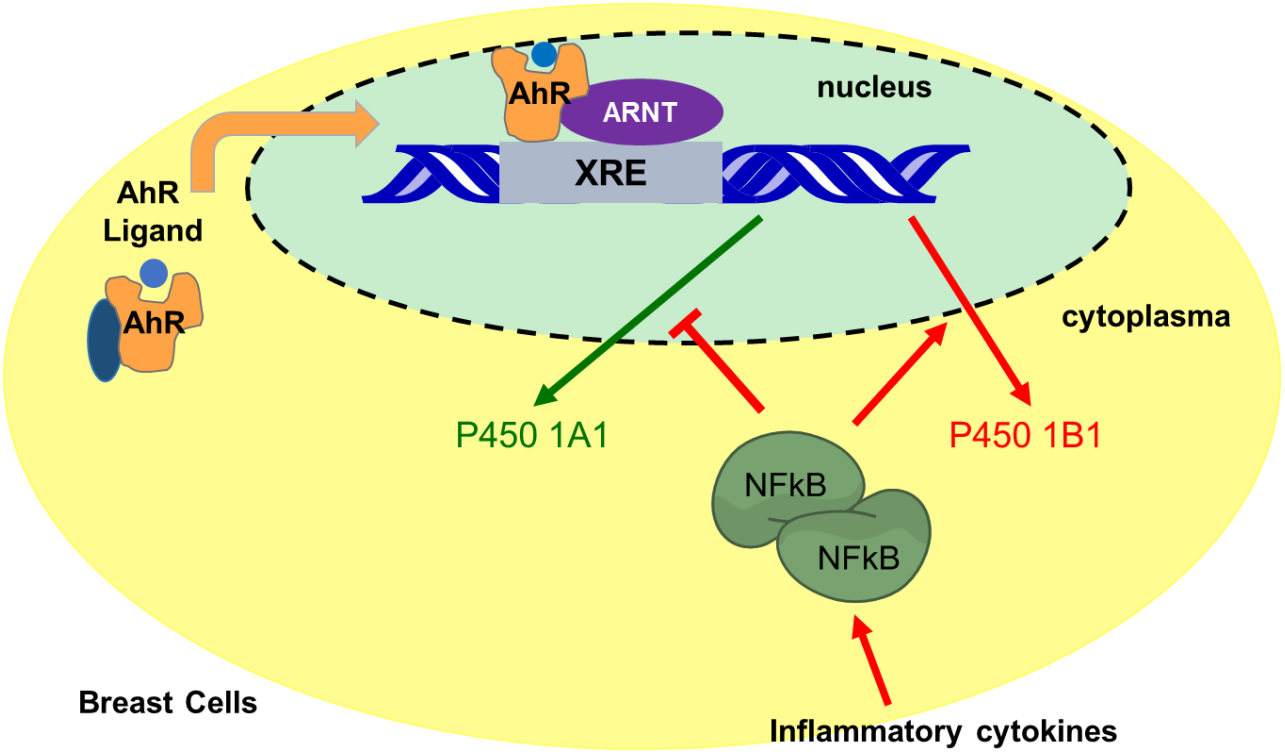


Figure 5. Crosstalk interference of P450 1A1 and 1B1 regulation by the inflammation pathway. Inflammatory cytokines activate NFκB signaling which induces the expression of P450 1B1 and limit the expression of P450 1A1.

1.5. Breast Cancer Prevention

Since breast cancer is the most common and second deadliest cancer in women in the United States, preventing breast cancer provides a significant benefit for society. Although several of the breast cancer risk factors could not be controlled (i.e., aging, race, genetic factors), continuous estrogen exposure can be an underlying cause of some of the risk factors. For example, late menarche and early puberty extend the time women are exposed to estrogen. Local estrogen production in adipose tissues is much more than that in normal tissues. Thus, controlling estrogen exposure or actions could potentially help prevent breast cancer.

Aromatase inhibitors (AI) like anastrozole and exemestane, reduce the amount of estrogen that women are exposed to, and have been used as first line therapy for breast cancer treatment (Dowsett et al., 2015). From a breast cancer prevention perspective, AIs were shown to inhibit both estrogen hormonal and chemical carcinogenesis pathways from *in vitro* studies (Yue et al., 2005), and reduce breast cancer risk by more than 50% in randomized clinical trials (Cuzick et al., 2014; Goss et al., 2011). However, AIs suffer from major side effects including potential bone loss and cardiovascular diseases with long term treatment.

Selective estrogen receptor modulators (SERMs), such as tamoxifen and raloxifene, are a class of compounds that inhibit ERα signaling in a tissue specific manner (Diez-Perez, 2006). Both tamoxifen and raloxifene have shown promising results in reducing ER positive breast cancer risks in multiple breast cancer prevention clinical trials (Brown and Lippman, 2000; Fisher et al., 1998; Vogel, 2016). However, SERMs are not used as

standard breast cancer prevention agents due to its risk in thrombosis and stroke (Freedman et al., 2011).

Estrogen metabolism modulation as a breast cancer prevention strategy has mostly been studied preclinically. P450 1B1 serves as a viable target for breast cancer prevention due to its important role in estrogen chemical carcinogenesis. Effects have been seen in breast cancer prevention through targeting P450 1B1. Compounds acting as P450 1B1 inhibitors (or down-regulators) such as resveratrol and beta-naphthoflavone, have been tested in *in vitro* experiments as an approach for breast cancer prevention (Chen et al., 2004; Chun et al., 2001; Peter Guengerich et al., 2003; Shimada et al., 1998). On the other hand, P450 1A1 mediated estrogen 2-hydroxylation acts as a competing benign pathway and has clinically been associated with reduced breast cancer risk (Fuhrman et al., 2012). The balance between the amount of parent estrogen, the benign 2-hydroxylation pathway, and the genotoxic 4-hydroxylation pathway plays a critical role in estrogen chemical carcinogenesis. Modulation of estrogen metabolism with less 4-hydroxylation and more 2-hydroxylation could help prevent breast cancer.

Phase II conjugation enzymes have been shown to be less active during carcinogenesis in catalyzing the inactivation and excretion of estrogens and estrogen metabolites; thus, upregulation of phase II enzymes is also a viable strategy to reduce estrogen carcinogenesis (Suzuki et al., 2011; Suzuki et al., 2003). Additionally, diet adjustment and intake of dietary supplements are considered another way to reduce breast cancer risk.

1.6. Botanical Dietary Supplements for Women's Health

1.6.1. Botanical dietary supplements use and research

The use of botanical dietary supplements in the US has been steadily increasing in the past decade (Bailey et al., 2011). After the publication of results from the Women's Health Initiative (WHI) study in 2002, the prescription of hormone therapy has dropped significantly over the years and stayed at a low level ever since (Jewett et al., 2014). In the study findings, breast cancer risk was significantly increased in postmenopausal women using the combined hormone therapy in comparison to placebo (Rossouw et al., 2002). As a result, women are looking for natural alternatives to traditional HT. Although neither safety, efficacy, nor standardization evidence is required by the Food and Drug Administration (FDA) prior to marketing of botanical dietary supplements (Fortin, 2008), botanical dietary supplements are being widely used in the United States with about 20% of adults reporting using at least one type of botanical dietary supplements (Bailey et al., 2011).

One of the major reasons for the use of botanical dietary supplements is for the natural relief of postmenopausal symptoms. Even though the effect on hot flash relief by black cohosh and red clover extracts cannot be compared with the Prempro group during a randomized clinical trial, these botanical extracts did reduce the severity of postmenopausal symptoms by about 50% similar to a placebo (Geller et al., 2009).

Studies on the health benefits of botanicals in various aspects have been extensively conducted in the last decade. Several clinical study results have linked vegetable consumption with cancer prevention potential (Freudenheim et al., 1996; Shannon et al., 2005). Particularly, botanical compounds such as lycopene, curcumin, sulforaphane, and resveratrol have been found and purified from plants with cancer

prevention activities (Kundu and Surh, 2008; Park and Surh, 2004; Surh, 2003). Sulforaphane isolated from broccoli was found to act on Nrf2/Keap1 protein complex and activate Nrf2 signaling which increases the production of phase II detoxification enzymes, especially NQO1 to help prevent carcinogenesis (Kensler et al., 2013; Shinkai et al., 2006). It has also shown promising activity in airborne pollutant detoxification from a broccoli sprout clinical trial in China (Egner et al., 2014; Kensler et al., 2012). Resveratrol has also shown activity in pre-clinical and clinical studies for cancer prevention and treatment through acting on proliferation and apoptosis pathways, inflammation pathways, as well as different stages of carcinogenesis (Aggarwal et al., 2004; Athar et al., 2007; Bishayee, 2009).

Among botanicals studied for estrogen chemical carcinogenesis, resveratrol from grapes has been mostly studied and reported with promising activity. *In vitro* studies with ER (-) non-tumorigenic breast MCF-10F cells showed that resveratrol inhibited estrogen induced colony formation, decreased estrogen depurinating adduct formation, and reduced estrogen oxidative metabolism (Lu et al., 2008; Zahid et al., 2008; Zahid et al., 2011). Potential mechanisms for the reduction of the estrogen chemical carcinogenesis were through the inhibition of hydroxylation of estrogen to catechols via P450 1A1 and 1B1 enzyme downregulation (Chen et al., 2004) as well as the ability to scavenge ROS and inhibit NF-kB signaling (Leonard et al., 2003). Sulforaphane from broccoli also reduced estrogen depurinating adduct formation partially through upregulation of NQO1 and GST in MCF-10A cells (Yang et al., 2013).

1.6.2. Models for estrogen chemical carcinogenesis prevention study

To study the effect of estrogen chemical carcinogenesis, various *in vitro* models have been used including using liver microsomes, recombinant proteins and cell lines. While cell free systems are better to study mechanisms, cell models are mostly used for studying modulation of estrogen chemical carcinogenesis. The MCF-10 cell line is an immortalized non-tumorigenic breast epithelial cell line that does not express ER-α. Two sub cell lines have been characterized from the initial culturing. MCF-10A is derived from the adhesive cells and MCF-10F from the floating cells (Soule et al., 1990). Given its non-tumorigenic character and negative ER-α status, MCF-10 cells have been frequently used as an *in vitro* model to study estrogen chemical carcinogenesis (Chen et al., 2005; Lu et al., 2007). Additionally, MCF-7 cells have also been used previously in the study of estrogen chemical carcinogenesis due to its much higher sensitivity upon AhR activation (Huang et al., 2011; Spink et al., 1998; van Duursen et al., 2003).

Various *in vivo* studies have also been done in regards of estrogen chemical carcinogenesis. Long term estrogen carcinogenesis study is done with the ACI rat model, which is an animal model that is sensitive to estrogen induced mammary carcinogenesis (Shull et al., 1997). However, as the hormonal pathway and chemical pathway cannot be separated out in the *in vivo* system, studies on the *in vivo* estrogen chemical pathway were limited. Clinical studies have used serum estrogen and estrogen metabolites as biomarkers for breast cancer risk evaluation (Gaikwad et al., 2008; Pruthi et al., 2012).

1.6.3. Licorice

Licorice (*Glycyrrhiza*) belongs to the Fabaceae family and has traditionally been used for bacterial/viral infections and anti-inflammation purposes (Hajirahimkhan et al.,

2013a; Kao et al., 2014). There are three medicinal licorice species commonly used in botanical dietary supplements: *Glycyrrhiza glabra* (GG)*, Glycyrrhiza uralensis* (GU) and *Glycyrrhiza inflata* (GI), with GG being most common in the United States (Hajirahimkhan et al., 2013a; Kondo et al., 2007; Park et al., 2014; Upton et al., 2010). Isoliquiritigenin (LigC) and liquiritigenin (LigF) are the major bioactive compounds discovered in licorice across species (Figure 6). LigC undergoes cyclization to form LigF in physiological conditions. LigC showed activity in inducing NQO1 as a chemopreventive compounds, and LigF showed ERβ selective estrogenic activity (Cuendet et al., 2010; Cuendet et al., 2006; Gong et al., 2014). Licochalcone A (LicA) from licorice is a unique chalcone that is only found in GI but represents more than 5% in the crude extract (Farag et al., 2012). LicA is also a Michael acceptor, but does not have the self-cyclizing capability. LicA has been found to have anti-inflammatory activity through covalent inhibition of iKK and NF-kB signaling (Fu et al., 2013; Funakoshi-Tago et al., 2010b; Furusawa et al., 2009). Studies have also shown the ability of LicA in inducing NQO1 in MCF-10A cells (Hajirahimkhan et al., 2015). The chemical profile differences from the three licorice species as well as the reported chemopreventive and anti-inflammatory activities from its bioactive compounds make licorice a good botanical candidate to study.

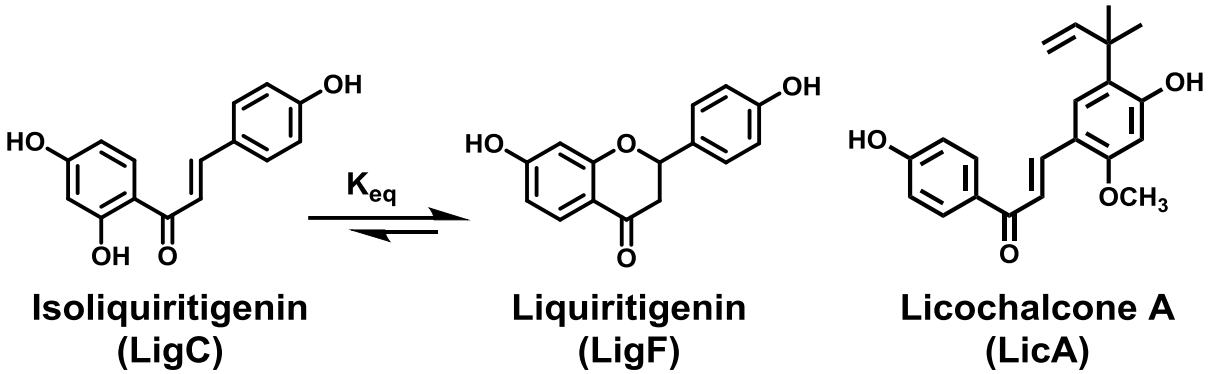


Figure 6. Key bioactive marker compounds in licorice.

1.6.4. Hops

Hops (*Humulus lupulus*) is a species in the Cannabaceae family and has been traditionally used as a sleep aid and more recently as a surrogate for hormone replacement therapy (Aghamiri et al., 2015; Keiler et al., 2013). Hops has also been frequently used in the beer industry as a flavoring agent. Hops contains a series of prenylated flavonoids and chalcones, which were found to be responsible for most of its biological activities discovered. 6-Prenylnarigenin (6-PN), 8-prenylnarigenin (8-PN), isoxanthohumol (IX), and xanthohumol (XH) are the major prenylated compounds in hops (Figure 7) (Legette et al., 2014; Nikolic et al., 2005). Hops has been previously shown to have estrogenic and chemopreventive activities through the activity of 8-PN and XH, respectively. 8-PN showed nM potency in ER-α activation representing the most potent phytoestrogen known to date (Chadwick et al., 2004; Milligan et al., 2000). 8-PN was also found to be an aromatase inhibitor with nM activity (Monteiro et al., 2006). XH is the major

prenylated compound in hops and was found to induce NQO1 from both *in vitro* and *in vivo* studies (Dietz et al., 2013; Gerhäuser et al., 2002; Stevens and Page, 2004). 6-PN and IX have been studied mostly in comparison with 8-PN and XH, but did not show much activity from previous studies. Since hops contains bioactive compounds with ERα activity and NQO1 induction activity with its unique prenylated compounds, it is also interesting to see if hops and its compounds have activity in modulating estrogen chemical carcinogenesis.

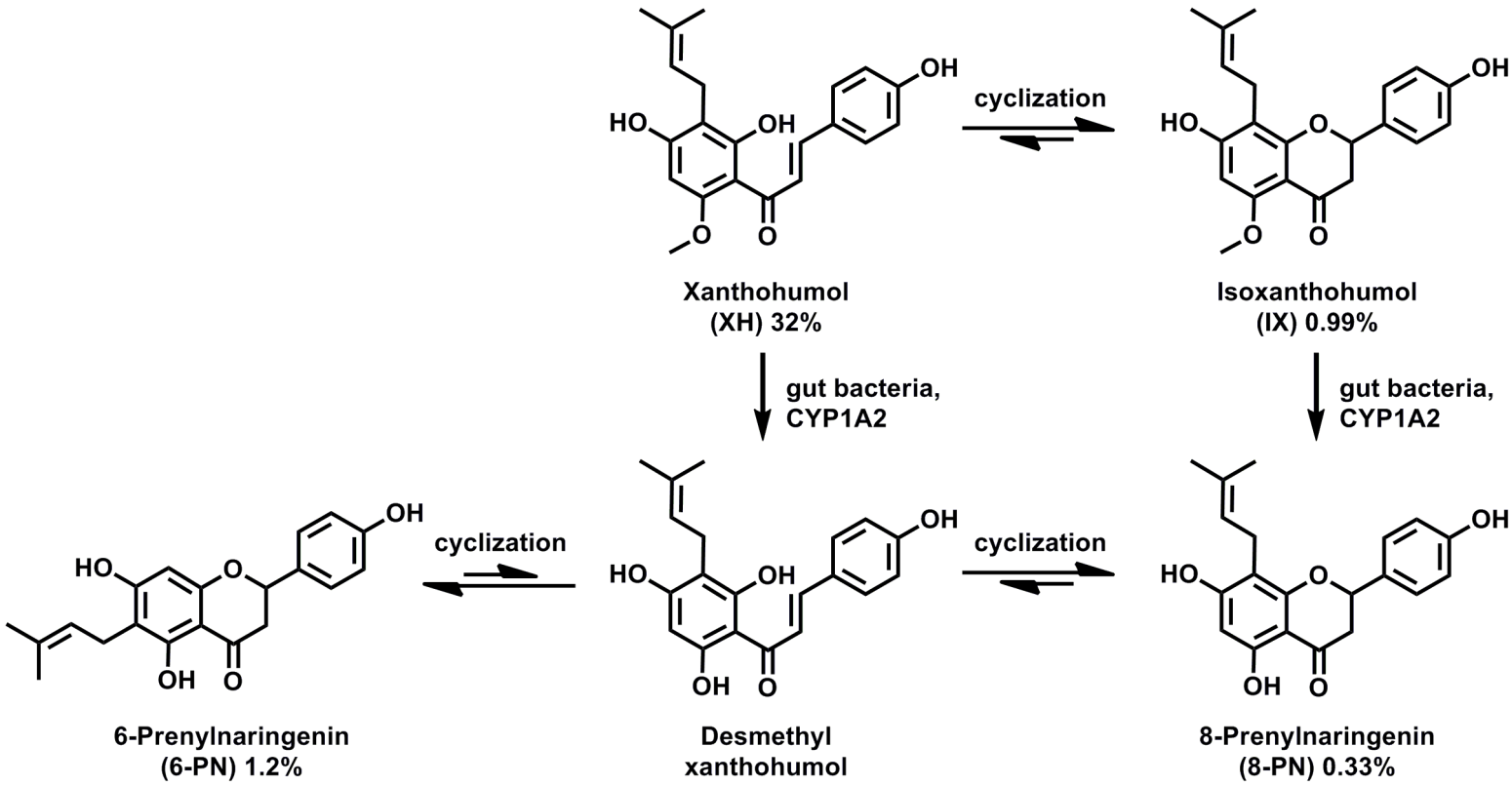


Figure 7. Key bioactive compounds in hops.

1.7. Specific Aims for the Study

The goal of this study was to determine the effects of botanical dietary supplements on estrogen chemical carcinogenesis and to discover beneficial botanicals and bioactive compounds that could modulate estrogen oxidative metabolism to potentially reduce the risk of breast cancer. It is ***hypothesized*** that botanicals could modulate estrogen oxidative metabolism to produce less 4-hydroxylation products and more 2-hydroxylation products so as to lower the risk of estrogen chemical carcinogenesis. Since licorice and hops are two of the most studied botanicals with anti-inflammatory and chemopreventive activities, this study is focused on characterizing the activity of these two botanicals on estrogen chemical carcinogenesis. Two aims were proposed:

I. Optimization of LC-MS/MS method for the detection of estrogen oxidative metabolism.
II. Evaluation of botanical dietary supplements (licorice and hops) and their bioactive compounds on estrogen chemical carcinogenesis.

In the present study, authenticated botanical extracts and pure compounds were tested in non-tumorigenic breast epithelial (MCF-10A) and tumorigenic ER+ breast (MCF-7) cells. The influence on estrogen oxidative metabolism was quantified and enzyme activity and expression measured for its mechanism of action. ACI rat model was adopted to study promising botanicals and bioactive compounds on the effect of estrogen oxidative metabolism *in vivo*. These studies showed beneficial effects of licorice and hops extract on estrogen oxidative metabolism modulation, which point out the potential of licorice and hops botanical dietary supplements on breast cancer prevention.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

All chemicals and reagents were purchased from Fisher Scientific (Hanover Park, IL) or Sigma (St. Louis, MO), unless otherwise stated. Liquiritigenin and isoliquiritigenin were obtained from ChromaDex (Irvine, CA). IKK inhibitor VII was purchased from EMD Millipore (Billerica, MA), and 2-methoxyestrone ($MeOE_1$)-1,4,16,16-d4 was obtained from CDN isotope (Pointe-Claire, Quebec). E_2, 2- and 4-$MeOE_1$ reference compounds were acquired from Steraloids Inc. (Newport, RI). TNF-α and IFN-γ were purchased from R&D systems (Minneapolis, MN). Cell media were obtained from Invitrogen (Carlsbad, CA).

2.1.1 Plant material, extraction, and characterization

Glycyrrhiza glabra was purchased from a local supplier in Chinatown (Chicago, IL) and *Glycyrrhiza uralensis* from Indiana Botanical Garden. *Glycyrrhiza inflata* was collected in Xinjiang province, China, in 2012. All plant materials were identified through a series of macroscopic and microscopic analyses, comparing the plants with voucher specimens from the Field Museum in Chicago, IL. DNA authentication (Kondo et al., 2007) confirmed the botanical identity of all *Glycyrrhiza* species. Standardized licorice extracts were prepared as previously reported (Hajirahimkhan et al., 2013a). The amount of LigC/LigF equivalents was determined via UHPLC-UV (Figure 8). For the analysis, each licorice extract was prepared at 10 mg/mL in 100% MeOH HPLC grade. All samples were analyzed on a Kinetex XB-C18 column and eluted with a gradient composed of H_2O 0.1% formic acid (A) and ACN 0.1% formic acid (B) (Simmler et al., 2013b). Except for licochalcone A, all compounds were isolated from GU (Simmler et al., 2013a; Simmler et

al., 2013b). A standard curve containing the following 11 reference standards was used for their quantitation in each extract. The area under the curve (AUC) was taken at 360 nm for all chalcones (isoliquiritin, isoliquiritin apioside, licuraside, LigC, licochalcone A), and at 275 nm for all flavanones (liquiritin, liquiritin apioside, liquiritigenin 7-*O*-apiosylglucoside, LigF) (Figure 9). Quantitative results obtained for each LigF glycoside were corrected by a factor corresponding to [molecular weight (MW) of LigF]/[MW of LigF glycoside], thereby leading to their concentration as LigF equivalents; LigC equivalents were likewise quantitated. The purity of each investigated isolated compound was determined by quantitative 1D ^{1}H NMR using the 100% method (Pauli et al., 2005) and yielded the following purity percentages (in % w/w): LicA 99.3% (ratio *trans/cis* = 93/7), LigF 96.6%, and LigC 98.6%.

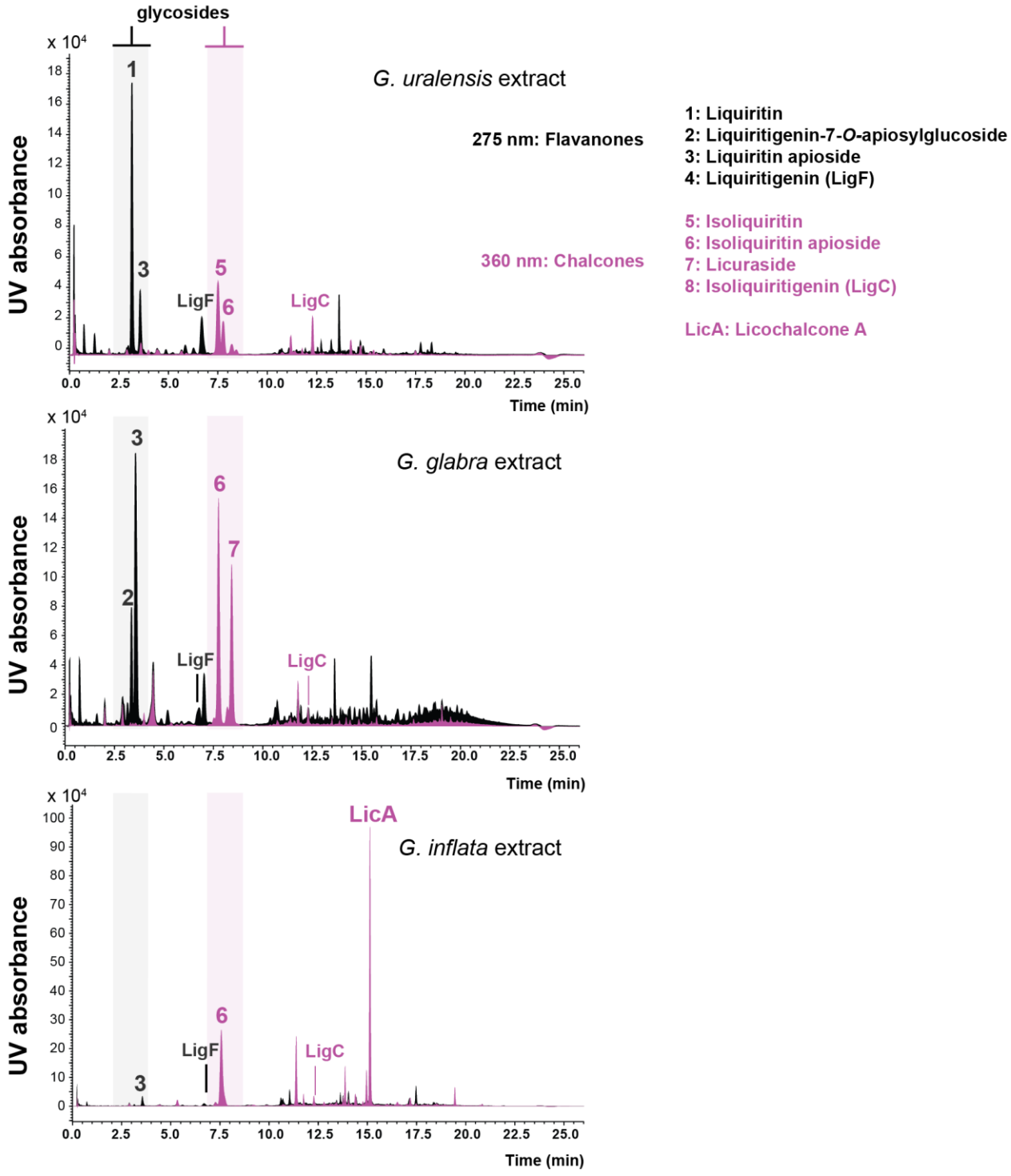
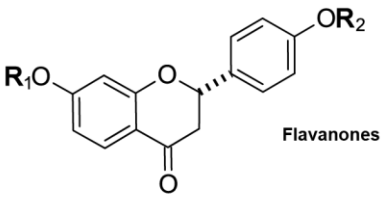


Figure 8. Chemical profiles of GG, GU and GI, as measured by UHPLC-UV. Liquiritin (Liq) Liquiritin apioside (LiqA) and liquiritigenin -7-O-apiosylglucoside (7LiqA) are all LigF equivalents, which share the same core aglycone LigF structure. Isoliquiritin (ILiq), isoliquiritin apioside (ILiqA), licuraside (Licur) are all considered LigC equivalents, which share the same core aglycone LigC structure.

LigF Equivalents

1	Liquiritin	R_1:H	R_2:β-Glc
3	Liquiritin apioside	R_1:H	R_2:β-Api(1→2)β-Glc
2	Liquiritigenin-7-O-Apiosylglucoside	R_1:β-Api(1→2)β-Glc	R_2:H
4	Liquiritigenin (LigF)	R_1:H	R_2:H



LigC Equivalents

5	Isoliquiritin	R_1:H	R_2:β-Glc
6	Isoliquiritin apioside	R_1:H	R_2:β-Api(1→2)β-Glc
7	Licuraside	R_1:β-Api(1→2)β-Glc	R_2:H
8	Isoliquiritigenin (LigC)	R_1:H	R_2:H

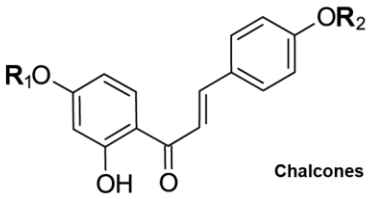


Figure 9. Structures of LigC and LigF equivalents.

The ethanol extract of botanically authentic spent strobili of *Humulus lupulus* was obtained from Hopsteiner (Mainburg, Germany, and New York, NY) and standardized to the prenylated polyphenol marker compounds 6-PN, 8-PN, IX, and as previously described (Figure 7) (Krause et al., 2014; Ramos Alvarenga et al., 2014). In short, standardization involved characterization by LC-UC, LC-MS/MS, and quantitative ^{1}H-NMR (qHNMR). The same extract has been used in a Phase I clinical trial in postmenopausal women (van Breemen et al., 2014). The concentrations of the four marker compounds in this extract were: 1.2% 6-PN, 0.33% 8-PN, 0.99% IX, and 32% XH.

2.2 Cell Culture and Conditions

MCF-10A, MCF-7, HepG2, and RAW 264.7 cells were obtained from American Type Culture Collection (Manassas, VA). MCF-10A cells were maintained in 1:1 Dulbecco's Modified Eagle's Medium (DMEM/F12) and supplemented with 1% penicillin-

streptomycin, 5% horse serum, cholera toxin (0.1 µg/mL), epidermal growth factor (20 ng/mL), hydrocortisone (0.5 µg/mL), and insulin (10 µg/L) . Additional media for MCF-10A cells were prepared with DMEM/F12 media without phenol red and supplemented with 5% charcoal-dextran treated horse serum at 37 °C with 5% CO_2 as described. MCF-7 cells were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum, 1% glutamax, 1% AB/AM, 1% non-essential amino acids, and 3 µg/mL insulin. HepG2 cells were maintained in DMEM/F12 medium with 10% FBS and 1% penicillin-streptomycin. RAW 264.7 cells were maintained in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin. All experiments were done with cells under 15 passages in phenol-red free media supplemented with charcoal stripped serum and the same other ingredients.

2.3. Analysis of Estrogen Oxidative Metabolism Using LC-MS/MS

2.3.1. *In vitro* and *in vivo* sample preparation

The 2- and 4-methoxyestrone (2- and 4-$MeOE_1$) metabolites were measured as indicators of the 2- and 4-hydroxylation metabolic pathways using modifications of literature methods (Figure 3) (Hemachandra et al., 2012; Xu et al., 2007a). Estradiol and all the standard compounds of estrogen metabolites were obtained from Steraloids Inc. (Newport, RI). Cells were estrogen starved for 24 h before being seeded into 6-well plates. MCF-10A/MCF-7 cells were plated 24 h before treatment, and cells were incubated with E_2 (1 µM) in the presence and absence of the treatment and vehicle control for 2 days. The cell media were then collected and spiked with the internal standard 2-methoxyestrone-d_4, obtained from CDN isotope (Pointe-Claire, Quebec), and ascorbic acid (2 mM). After

mixing, the collected cell media were extracted twice (x 4 ml) with CH_2Cl_2 (DCM). Organic layers were combined and dried under a gentle stream of nitrogen.

In vivo serum samples were either directly extracted with DCM or extracted after glucuronidase and sulfatase hydrolysis. However, with samples directly extracted with DCM, The amount of methoxyestrone metabolite was below limit of quantitation. After hydroxylation, both parent estradiol and methoxyestrone metabolite were observed with good signal to noise level (Figure 18). Only 2-MeOE1 was shown due to the specificity of rat P450 1B1 enzyme in favoring catalyzing the 2-hydroxylation of estrogen (Nishida et al., 2013). Thus, 2-MeOE1 was used as a marker for estrogen oxidative metabolism. To analyze, serum samples (150 µl) were incubated at 37 °C for 4 h after adding glucuronidase & sulfatase hydrolysis buffer (300 µl) and internal standard 2-methoxyestrone-d_4. After incubation, samples were extracted twice (x 1 ml) with DCM. The organic layers were combined and dried under a stream of nitrogen.

2.3.2. Analysis of estrogen methyl ethers as their dansylated derivatives

Derivatization was performed with dansylation reaction to put dansyl group on estrogen and estrogen metabolites to improve ionization for mass-spec analysis. $NaHCO_3$ buffer (0.1 M, pH 9.5) (100 µL) and dansyl chloride in acetone (1 mg/mL) (100 µL) were added to the extracted sample and incubated in a 60 °C water bath with agitation for 5 min, and kept on ice thereafter. Derivatized samples were analyzed by positive ion electrospray tandem mass spectrometric method using an Agilent 1200 series nano flow LC system (Agilent Technologies, Aanta Clara, CA) coupled to a AB SCIEX QTRAP™ 5500 System (AB SCIEX, Framingham, MA).

The liquid chromatography separation was carried out with a 50 mm × 3 mm i.d. Acquity BEH C-18 column packed with 1.7 µm particles (Waters, Milford, MA) and maintained at 40 °C. A total of 5 µL of each sample was injected into the column. The mobile phase (flow rate of 500 µL/min) consisted of 30% MeOH in water with 0.1% (v/v) formic acid as solvent A and 0.1% (v/v) formic acid in MeOH as solvent B. The initial conditions were set at 45% B. The liquid chromatographic gradient was held at 45% B for 1 min followed by a linear gradient to 80% B in 8 min and was held 1 min before equilibrating with initial conditions for 1 min. Mass spectrometer parameters were optimized as follows: the ion spray voltage was 5.5 kV; the source temperature was 500 °C; the curtain gas was 30 psi; the ion source gas 1 (nebulizer) was set to 40 psi and the ion source gas 2 (turbo gas) to 50 psi; the dwell time for each transition was 50 ms. Multiple reactions monitoring transitions were selected as follows: 534.4 – 171.2 for the detection of dansylated $MeOE_1$ and 538.4 – 171.2 for dansylated $MeOE_1$-d_4. MS fragmentation pattern of dansylated 4-$MeOE_1$ is shown (Figure 10). Quantitation was performed using the Analyst software (Applied Biosystems, Forster City, CA) in SRM mode.

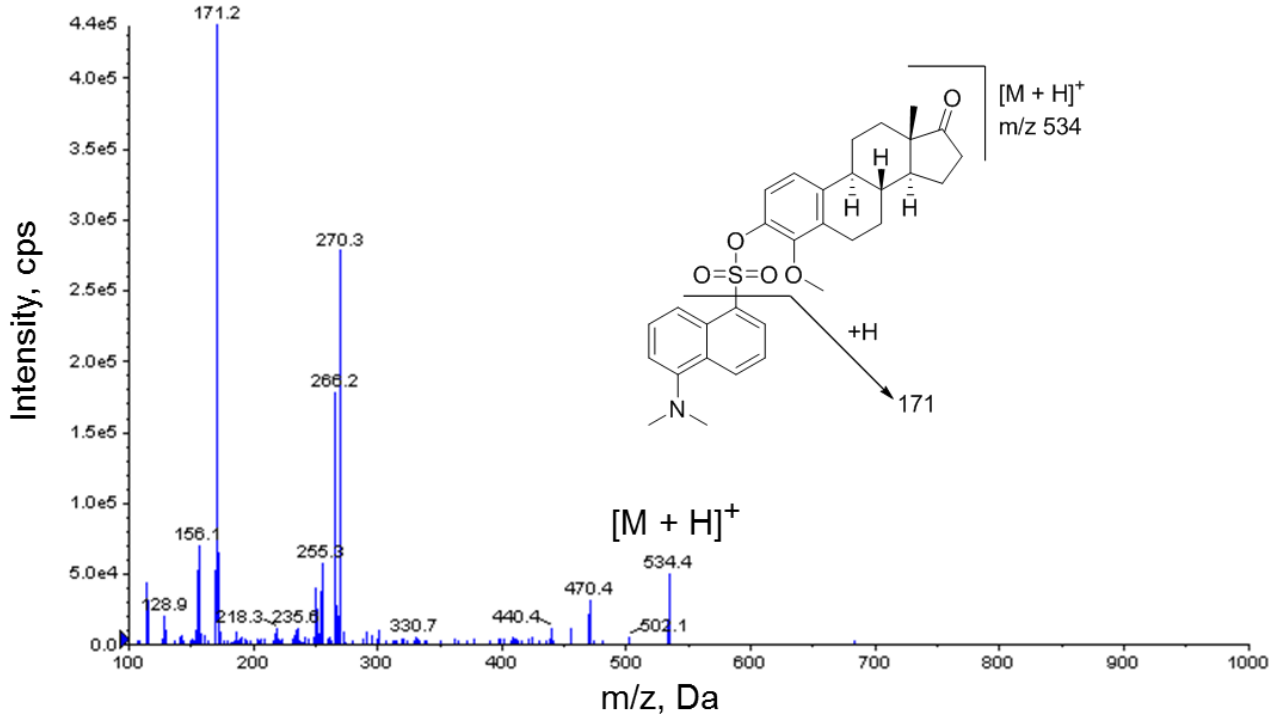


Figure 10. MS fragmentation pattern of dansyl-4-MeOE1. Representative positive ESI fragmentation mass spectra for parent ion dansyl-4-MeOE1 was shown. Parent ion Q1 with m/z of 534.4 was captured and scanned for Q3 product ions formation and the most intensive product ion with m/z of 171.2 was selected to monitor in the LC-MS/MS.

2.4. Griess Assay for Detection of NO2-

This assay to detect NO2- levels produced from cells was performed according to a previous method (Dirsch et al., 1998; Dunlap et al., 2008) with some minor modifications. RAW 264.7 macrophage cells were plated at a density of 12 x 104 cells/mL in 96-well plates overnight. Cells were treated with the agent for 1 h, prior to addition of LPS (1 µg/mL) for 24 h. Griess reagent (0.5% sulfanilamide, 0.05% (N-1-naphthyl) ethylenediamine dihydrochloride) in 2.5% w/w H3PO4 was prepared, and media (50 µL)

was collected from cells and incubated with the Griess reagent (150 µL) for 30 min in 96-well plates at RT. Absorbance was then measured at 530 nm, and concentrations were calculated based on a $NaNO_2$ standard curve.

2.5. EROD Assay for P450 1A/1B Activity

Ethoxyresorufin-O-deethylation (EROD) activity was measured both in MCF-10A/MCF-7 cells and with recombinant P450 1A1 and 1B1 proteins as previously described for enzyme modulation and inhibition (Andrieux et al., 2004; Trapani et al., 2003). For enzyme modulation, MCF-10A/MCF-7 cells were plated in 96-well plates for 24 h. Compounds were added with and without 1 h post addition of TCDD (10 nM) for 48 h. Cells were washed twice with PBS and incubated with ethoxyresorufin (2.5 µM) and salicylamide (1.5 mM) at 37 ºC. For enzyme inhibition, MCF-10A cells pretreated with TCDD for two days were washed with PBS and pre-incubated with test compounds for 5 min at 37 ºC. Subsequently, ethoxyresorufin (2.5 µM) and salicylamide (1.5 mM) were added. Fluorescence was measured every min with excitation at 530 nm and emission at 590 nm for 25 min with a Bio-Tec Synergy H4 Hybrid Multi-Mode Microplate Reader (Winooski, VT).

For the recombinant protein inhibition assay, recombinant P450 1A1 and 1B1 protein with reductase were purchased from BD Biosciences (Woburn, MA). 0.15 pmole of P450 1A1 or 0.8 pmole of P450 1B1 per well was pre-incubated with test compounds or 2,3',4,5'-tetramethoxystilbene (TMS) for 5 min at 37 °C in 50 mM potassium phosphate buffer (pH = 7.4) with 1 mM NADPH before adding ethoxyresorufin (2.5 µM). Negative controls were done in parallel without NADPH. Fluorescence signals were check to make

sure it was not affected by these compounds in the inhibition experiments. The reaction was initiated by adding 7-ethoxyresorufin solution in potassium phosphate buffer to a final concentration of 2.5 μM. IC_{50} and apparent Ki (Ki') values were calculated. EROD activity was measured as resorufin formation by fluorescence with excitation at 530 nm and emission at 590 nm every minute for 25 min at 37 °C using BioTek's Synergy H4 Multi-Mode reader. Fluorescence with 7-ethoxyresorufin as substrate was linear for more than 15 min and the reaction rate was determined from the slope of the linear regression curves plotted with data points measured in the first 15 min. A resorufin standard curve was used to calculate the P450 1A/1B activity.

2.6. Quantification of P450 1A1/1B1 mRNA expression via qPCR

MCF-10A/MCF-7 cells at a density of 2.5×10^5 cells/mL were plated in 6-well plates and incubated with treatments for 24 h. The total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA) according to the product instructions. RNA was reverse transcribed according to Invitrogen's SuperScript® III First-Strand Synthesis System for RT-PCR. The resulting cDNA (2 μL) was used for real-time PCR quantification using the *StepOnePlus™ Real-Time PCR System* (Applied Biosystems, Carlsbad, CA). Taqman gene expression master mix and P450 1B1 primer with FAM/MGB probe (Applied Biosystems, Carlsbad, CA) were added to a 96-well reaction plate with cDNA. Real-time quantitative PCR consisted of one cycle of 50°C for 2 min and 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Data were analyzed with the comparative C_T ($\Delta\Delta C_T$) method, using HPRT1 as the endogenous control. The results are expressed as fold difference in reference to the HPRT1 control.

2.7. Luciferase Assay for Xenobiotic Response Element (XRE) Activation

HepG2 and MCF-7 cells were plated in 12-well plates overnight and cells were transfected at 70% confluency with luciferase and renilla plasmids (Promega, Madison, WI), XRE pGL4.43 luciferase plasmid (1 µg) and pRL-TK (500 ng), using Lipofectamine® 2000 reagent (Invitrogen, Grand Island, NY) for 6 h. After 6 h transfection, cells were treated with hop extract/compounds with and without the presence of TCDD (10 nM) for 24 h and lysed with passive lysis buffer. Lysates were centrifuged and analyzed for luciferase activity according to Promega's Dual-luciferase® Reporter Assay System protocol using the FLUOstar OPTIMA luminometer (BMG Labtechnologies, Germany). The results were plotted as fold induction of control. The % of TCDD was obtained by setting TCDD's fold induction in the XRE-luciferase assay as 100%. The fold induction of compounds was divided by the TCDD response and multiplied by 100 to obtain the % of TCDD response.

2.8. MALDI-TOF Analysis of Interaction of LicA with AhR

Human recombinant AhR protein (10 µM) was incubated with vehicle or LicA (10 µM) at 37 °C with agitation for 30 min in PBS buffer. After the reaction, the reaction was mixed with equal volume of 10 mg/ml sinapinic acid/acentontrile. After the mixing, 1 µl of sample was pipetted on the metabolic plate (Bruker Daltonics Inc., Billerica, MA) and dried until crystals formed. The analysis was carried out with an Autoflex speed LRF MALDI-TOF mass spectrometer (Bruker Daltonics Inc.,). Spectra were obtained in positive linear mode and the plot was exported using the Flex Control software.

2.9. Animal Experiment

ACI rat model was used to study short term estrogen oxidative metabolism *in vivo*. Female ACI rats were purchased at 5 weeks of age and acclimated for about a week with the supply of phytoestrogen free diet (AIN-76A from Harlan Laboratories). The rats were divided into three groups with 6 rats per group: sesame oil as the vehicle, estradiol-benzoate at 1 mg/kg/day, and estradiol-benzoate at 1 mg/kg/day plus LicA at 80 mg/kg/day. Treatments were administered subcutaneously daily for 4 days at 6 weeks of age. After dosing, the rats were sacrificed by CO2 asphyxiation. Blood were collected and serum was prepared immediately after collection; mammary tissues, uterus, and liver were collected and snap frozen in liquid nitrogen and stored in -80 °C until analysis. The animal protocol complied with the Guide for the Care and Use of Laboratory Animals and all procedures were approved by UIC's Institutional Animal Care and Use Committee (Protocol No. 16-033).

2.10. NQO1 activity measurement

Around 400 mg of frozen liver samples were cut and homogenized in 0.25 M sucrose solution on ice. Homogenized samples were then centrifuged at 15, 000 x g for 1 h at 4 °C. The supernatants were collected and spiked with 0.1 M $CaCl_2$ in 0.25 M sucrose solution to a final concentration of 200 mM and kept at 0 °C for 30 min before centrifuging at 15, 000 x g for 30 min at 4 °C. The clear supernatant containing the cytosolic portion were collected and total protein was quantified using bicinchoninic acid assay (BCA). 5 µg of liver sample were used and NQO1 activity determined through measuring the reduction of MTT by menadiol reduced from menadione as previously described (Prochaska and Santamaria, 1988).

The supernatant was incubated with NQO1 reaction mixture solution for around 5 min and absorbance was measured at 610 nm. NQO1 was calculated after normalization to control. NQO1 reaction mixture stock solution was made as following: 7.5 mL of 0.5 M Tris-Cl (pH 7.4), 100 mg of bovine serum albumin, 1 mL of 1.5% Tween-20, 0.1 mL of 7.5 mM FAD, 1 mL of 150 mM glucose-6-phosphate, 90 µl of 50 mM NADP, 300 U of yeast glucose-6-phosphate dehydrogenase, 45 mg of MTT, and distilled water to a final volume of 150 mL. Menadione (1 µl of 50 mM menadione dissolved in acetonitrile per milliliter of reaction mixture) was added just before the reaction.

2.11. Measurement of LicA concentration in *in vivo* samples

After thawing at room temperature, serum (50 µL) was transferred to a 1.5 mL Eppendorf tube and mixed with 10 µL of acetonitrile (ACN) containing naringenin (500 nM) as an internal standard (IS). Liver and mammary tissue were weighed and homogenized in 70% aqueous methanol (containing 0.1% formic acid). Then 200 µL homogenate was taken and added with 20 µL naringenin (500 nM). Ice-cold ACN was added and the mixture was then centrifuged for 15 min at 13000 x g at 4 °C for protein precipitation. After centrifugation, 200 µL of the supernatant was transferred to a new Eppendorf tube and evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 100 µL of 20% ACN, 5 uL was injected into LC-MS/MS for analysis.

UHPLC-MS/MS analyses were carried out using a Shimadzu (Kyoto, Japan) LCMS-8060 triple quadrupole mass spectrometer equipped with a Shimadzu Nexera UHPLC system. Analytes were separated on a Waters (Milford, MA) Acquity UPLC BEH C18 2.1 × 50 mm column (1.7 µm particle size). For quantitative analysis, the gradient was as

follows: 0−1 min, hold 20% B; 1−3.5 min, from 20% to 45% B; 3.5-4.5 min, from 45% to 95% B; and hold at 95% B for 1.5 min, then back to 20% B for 2 min at a flow rate of 0.6 ml/min. Nebulizing gas flow: 2.5 L/min; heating gas flow: 10 L/min; interface temperature: 300 C; DL temperature: 250 C; heating block temperature: 400 C; drying gas flow: 10 L/min. The data were acquired using selected reaction monitoring (SRM) in electrospray (ESI) with polarity switching and the following SRM transitions (quantifier and qualifier): naringenin (m/z 273 > 153 and 273 > 147, internal standard); licochalcone A (m/z 339 > 121; 339 > 297). Dwell time is 15 msec for each transition.

2.12. In-Cell Western Blot Analysis of ER-α

Cells were estrogen starved for 3 days with stripped media before plating to 96 black plate with clear bottom. Cells were then treated 48 h after plating for 24 h. After treatment, cell media were dumped and cells were fixed with 100 µl of 4% formaldehyde solution for 20min. After fixation, cells were washed with PBST (0.1% triton x-100 in PBS) for 4 times with agitation and blocking buffer (LI-COR Biosciences) was added to the cells. After a 1.5 h incubation with low agitation at room temperature, the blocking buffer was discarded and primary anti-ERα antibody (Santa Cruz Biotechnology) at 1:1000 dilution was added and plate was kept overnight at 4 °C with low agitation. The cells were then washed with PBST (PBS Tween 0.1%) for 4 times and incubated with secondary antibody and celltag (1:2000) (LI-COR Biosciences) for 1hr at room temperature. After incubation, cells were washed again with PBST for 4 times and switched to PBS before imaging. In-cell western blot imaging and analysis were performed and scanned at both 700 nm and 800 nm with LI-COR Odyssey Sa Imaging Systems.

2.13. Statistics

The data were expressed as mean ± SEM from at least three independent experiments. Significance was determined using student t-test to compare two samples and one-way ANOVA with Dunnett's post-test to compare multiple samples with the control (*$p < 0.05$).

3. OPTIMIZATION OF A LC-MS/MS METHOD FOR THE QUANTITATION OF ESTROGEN OXIDATIVE METABOLISM

(Reprinted in part from: Dunlap TL, Wang S, Simmler C, Chen SN, Pauli GF, Dietz BM, and Bolton JL. “Differential effects of glycyrrhiza species on genotoxic estrogen metabolism: licochalcone A downregulates P450 1B1, whereas isoliquiritigenin stimulates it” *Chemical Research in Toxicology* 28(8), 1584-1594 (2015))

3.1. Methods for the Quantitation of Estrogen and its Metabolites

Quantitation of estrogen and estrogen metabolites has always been an interest for researchers due to the important biological effects of estrogens. There are several different types of quantitation methods developed, and have been replaced by improved methodologies due to the advancement of new technologies and instrumentations. Amongst the earliest methods, radio immunoassays (RIA) (Ball et al., 1979) and enzyme immunoassays (EIA) (Ziegler et al., 1997) utilized the specificity of antibody-antigen interaction to achieve detection of estrogens in low abundance. Although immunoassays are very sensitive and specific, detection of estrogen metabolites relies on well characterized antibodies, which are not available for every metabolite of interest. With the development of modern liquid chromatography (i.e. HPLC), multiple metabolites of interest can be detected in a single run, which increases efficiency and decreases variation. Furthermore, HPLC coupled with tandem mass spectrometer (LC-MS/MS) has provided the highest sensitivity and accuracy so far. LC-MS/MS is capable of targeting estrogen and estrogen metabolites and quantifying their amount through multiple reaction monitoring

(MRM). Thus, in this study, a LC-MS/MS method was used to quantify estrogen and its metabolites.

3.2. Derivatizations of Estrogen and Estrogen Metabolites for LC-MS/MS analysis

Although being the most sensitive and accurate, the analysis of molecules using mass spectrometry relies on the ionization of molecules of interest. However, estrogen and its metabolites are poorly ionizable due to their non-polar structure and lack of proton affinity atoms. Nevertheless, electrospray ionization (ESI) in negative mode has been tested with estrogen and its metabolites owing to its acidic phenol (Guo et al., 2008), but ion suppression has been reported for estrogens in biological matrices (Keski-Rahkonen et al., 2013) and the detection limit is often not enough for the quantitation of low abundance estrogen in various occasions.

To overcome the low ionization problem, derivatization reactions adding an easily ionizable chemical group on the estrogen phenol are frequently used to increase the ionization of estrogen. Derivatization reagents such as dansyl chloride, pyridine-3-sulfonyl (PS) chloride, and pocolinyl chloride or pocolinic acid are the most commonly used methods (Figure 11) (Blair, 2010). These derivatization reagents dramatically increased the sensitivity of the detection through the introduction of tertiary amine with reported lower limit of quantitation around 1 to 10 pg/ml (Xu and Spink, 2008; Xu et al., 2007b). More recently, derivatization reagents with quaternary amines have also been used for estrogen quantification and have shown higher sensitivity compared to traditional derivatization reagents (Khedr and Alandal, 2016; Wang et al., 2015; Yang et al., 2008a). In the current

study, dansyl chloride was used as the derivatization reagent for estrogen metabolite quantitation.

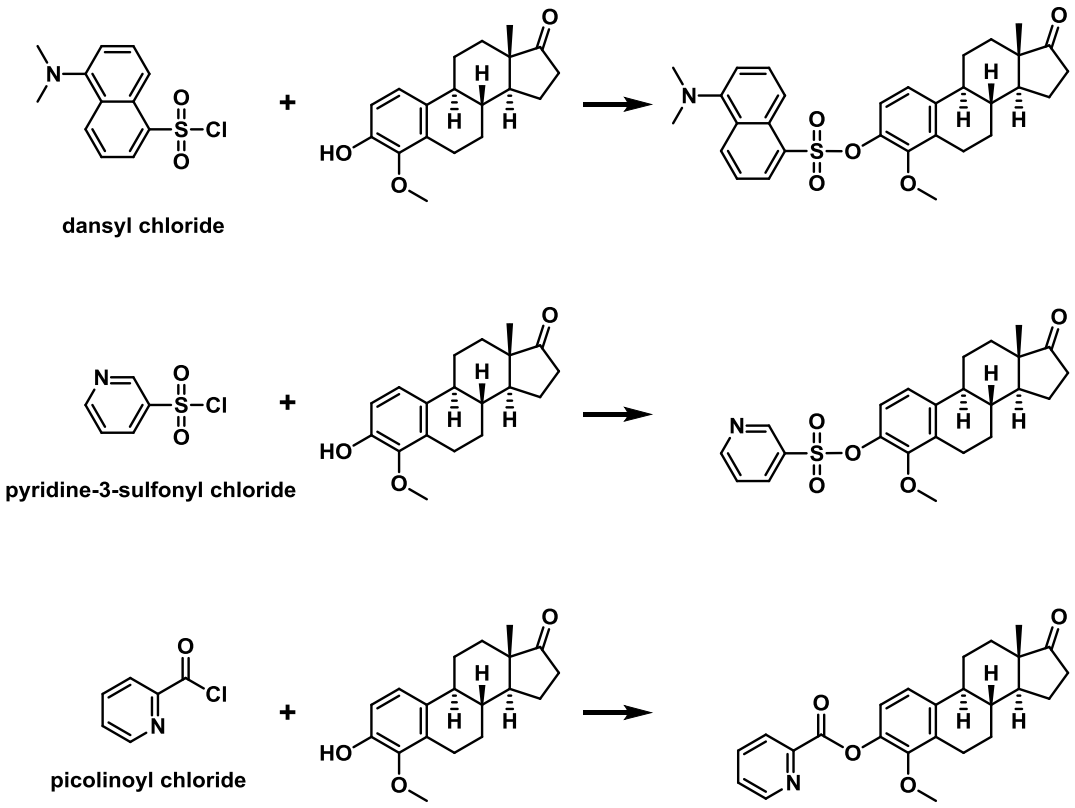


Figure 11. Common derivatization methods for estrogen metabolite analysis. Common derivatization methods for estrogen and estrogen metabolites using 4-MeOE$_1$ as an example. Dansyl chloride, pyridine-3-sulfonyl chloride, and picolinoyl chloride react with the estrogen 3-phenol to form conjugates with tertiary amine for increased ionization.

3.3. Optimization of the Assay for the Detection of Estrogen Metabolites.

3.3.1. LC-MS/MS and instrumentation improvement

For increased sensitivity and throughput, a more accurate and sensitive LC-MS/MS system (AB SCIEX QTRAP™ 5500 System) was used and it significantly enhanced sensitivity and signal to noise ratio (Figure 12). The coupled Agilent 1260 series LC system also allowed the use of UPLC columns and reduced the run time per sample from 32 min to 10 min, significantly increasing the throughput, which is necessary when dealing with a large amount of samples. In addition, the injection volume was reduced from 10 µl to 5 µl, minimizing the effect from the sample solvent. With this new method, the calibration curves were linear over a 100-fold concentration range (Figure 13). These methodological improvements gave enhanced sensitivity (>100-fold relative to previous studies) (Hemachandra et al., 2012) and allowed for rapid detection of estrogen methyl ethers, to determine the effect of extracts/compounds on metabolism.

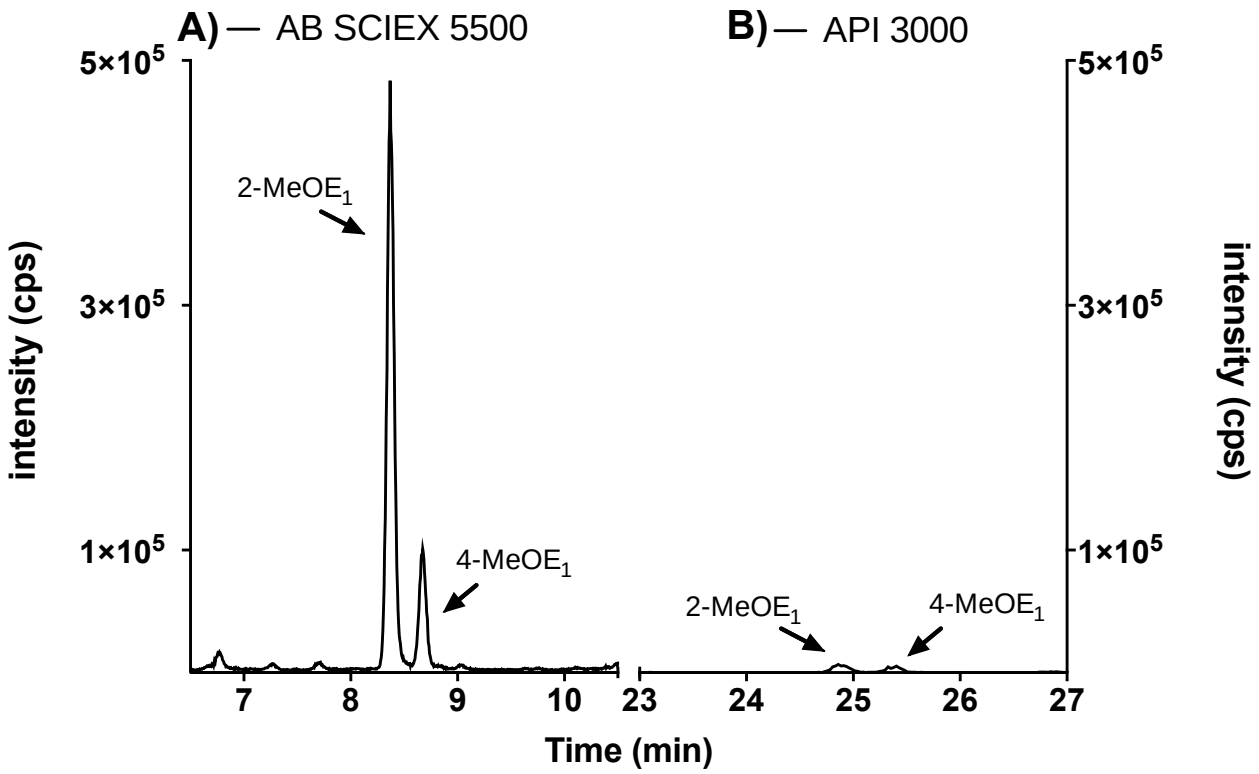


Figure 12. Representative $MeOE_1$ SRM chromatogram comparison. Representative chromatograms with transition 534.4 – 171.2 analyzing 2-$MeOE_1$ and 4-$MeOE_1$ metabolites showing improved sensitivity for A) current method compared to B) reference (Hemachandra et al., 2012). Note enhanced signal to noise for SCIEX 5500 QTRAP and shorter run times than the previous method using the API-3000 mass spectrometer. MCF-10A cells treated with E_2 (1 µM) were collected and analyzed for 2-$MeOE_1$ and 4-$MeOE_1$ metabolites as described in A) Chapter 2 and B) reference (Hemachandra et al., 2012).

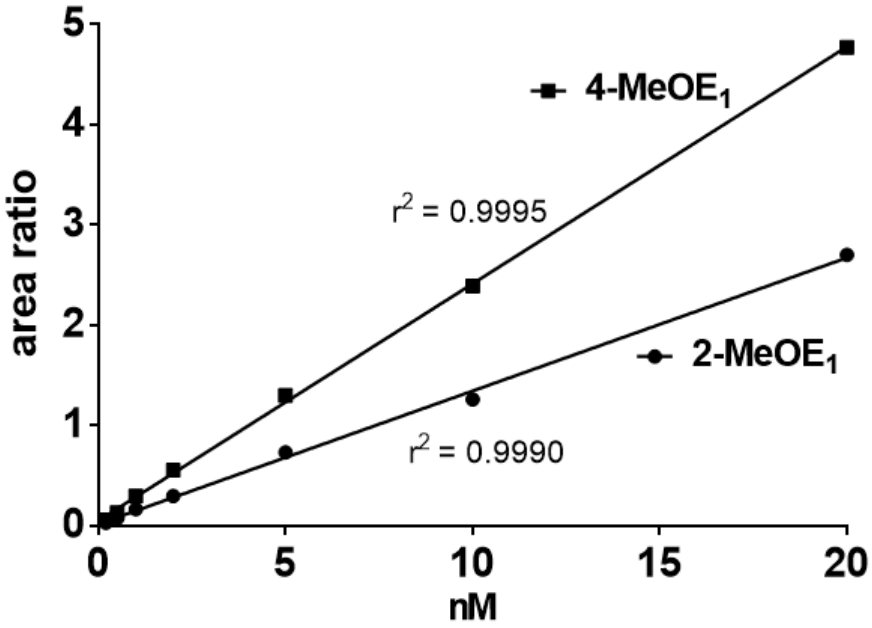


Figure 13. Standard curves for 2- and 4-MeOE$_1$. Standard solutions were prepared at different concentrations from 0.2 nM to 200 nM and internal standard at 50 nM. Calibration curves were plotted with the analysis results from cell media spiked with standard solutions and processed the same way as unknown samples.

3.3.2. *In vitro* experiment design and sample preparation optimization

Analysis of 2- and 4-MeOE$_1$ from MCF-10A cells was performed as described previously with several modifications to optimize the method (Hemachandra et al., 2012; Xu et al., 2007a). The previous method had an incubation time of 6 days with estradiol and treatment compounds, which required the refreshing treatment media every 3 days and storing treatment media until analysis. Since 5 mL of treatment media was added every 3 days, 10 mL of cell media was generated per sample. To perform liquid liquid extraction, the samples were lyophilized to approximately 2 mL to avoid handling larger volumes of samples.

To determine the optimum time to quantify estrogen metabolite levels, cells were treated with estradiol for different time periods and the amount of 4-MeOE$_1$ measured. Results showed that the amount of 4-MeOE$_1$ reaches plateau at 1 day (Figure 14), which allowed the assay time to be significantly shortened. Additionally, to take the enzyme modulation into consideration, the P450 1 enzyme induction measured by EROD assay indicated the highest induction was reached around 2 days (Figure 15). In the current method, a 2-day treatment time was adopted, thus, the treatment media did not need to be refreshed. This new method not only shortened the assay time from 6 to 2 days, but also significantly reduced the workload and reduced the sample volume by half. With the more sensitive LC-MS/MS method, the treatment media was further reduced from 5 mL to 3 ml, and the lyophilization step was also avoided.

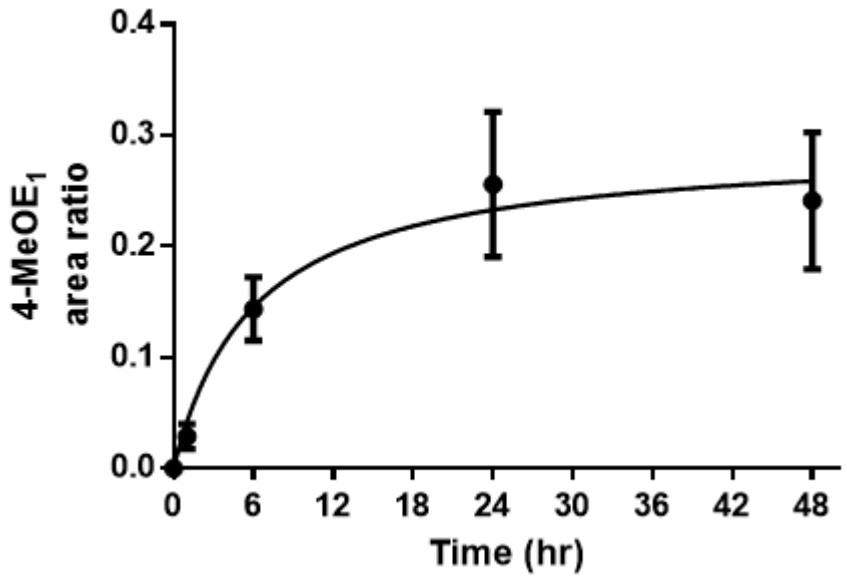


Figure 14. Time dependent formation of 4-MeOE$_1$ *in vitro*. MCF-10A cells were treated with 1 µM estradiol for 1 h, 6 h, 24 h and 48 h. The peak area ratio of 4-MeOE$_1$ to internal standard was plotted after normalization to the protein amount of the cells at different time points.

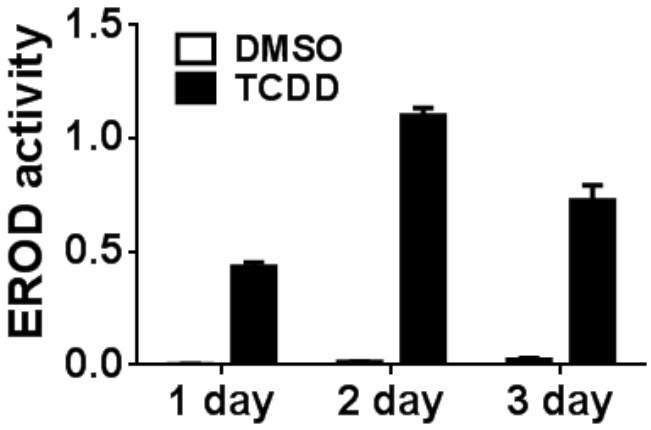


Figure 15. TCDD induced EROD activity was highest after 2 days of treatment. MCF-7 cells were treated with TCDD (10 nM) for 1 day, 2 day and 3 days. Cells were then measured for EROD activity as described in the Material and Methods section.

Additionally, the smaller sample volume also allowed for used of conical 15 mL glass tubes to perform liquid liquid extractions. Compared to the regular round bottom glass tubes, the extraction efficiency also increased from around 50% to 80% (Figure 16). Along with the improvement in the LC-MS/MS run time and sensitivity, and the optimization with treatment time and volume, the assay for quantitation of estrogen oxidative metabolism is much more robust than the previous method.

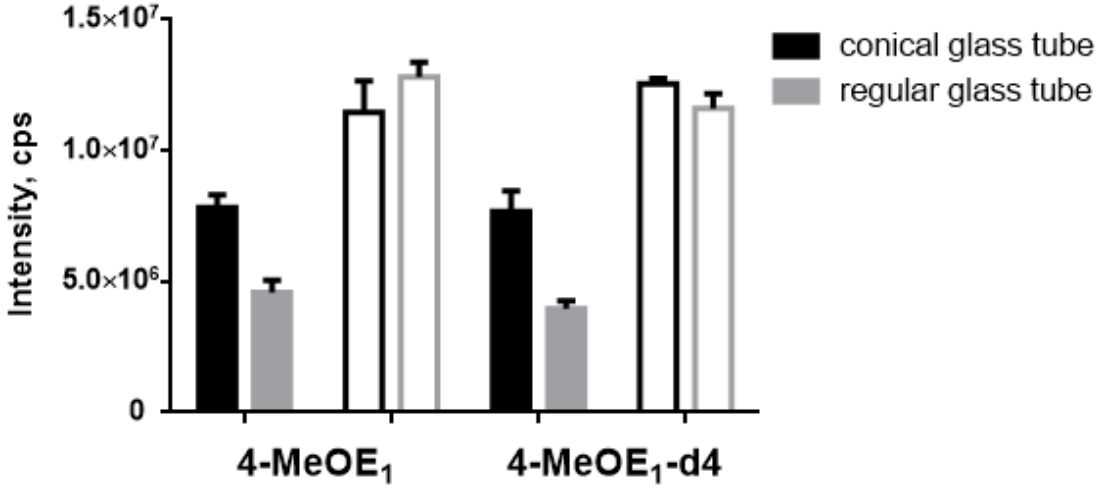


Figure 16. Increased extraction efficiency from the use of conical glass tube. Either 4-MeOE$_1$ or internal standard 4-MeOE$_1$-d4 was spiked into cell media matrix before (filled bars) and after extraction (empty bars) using conical or regular round bottom glass tubes. The samples were then processed for LC-MS/MS analysis and the peak area count was graphed.

3.3.3. *In vivo* experiment design and sample analysis

Catechol estrogens have been shown to induce DNA damage in the rat mammary tissues *in vivo* (Zhang et al., 2001). The ACI rat is an animal model for estrogen carcinogenesis, due to its high sensitivity towards estrogen induced mammary tumorigenesis (Shull et al., 1997). It has also been used previously to monitor formation of estrogen quinone DNA adducts in the mammary tissue (Li et al., 2004). To assess the *in vivo* effect of botanical extracts and bioactive compounds on estrogen oxidative metabolism, ACI rats were used and a model and a short term *in vivo* estrogen oxidative metabolism assay was designed.

To select a time point for the analysis of estrogen metabolites, ACI rats were dosed with estradiol-benzoate at 1 mg/kg subcutaneously in sesame oil and sacrificed at 0 h, 1 h, 4 h, and 24 h. The whole blood was collected and serum was prepared from the blood sample. Due to the significant extent of phase II metabolism *in vivo*, direct analysis of samples prepared without glucuronidase and sulfatase hydrolysis did not show enough signal for quantitative measurement (Figure 17). However, samples that were hydrolyzed with glucuronidase and sulfatase showed a time dependent increase of 2-MeOE$_1$ with the highest level detected at 24 h, yet 4-MeOE$_1$ formation was not observed. This result was due to the species difference between rat and human P450 1B1 enzyme, because rat P450 1B1 mainly catalyzes the estrogen 2-hydroxylation metabolism (Nishida et al., 2013).

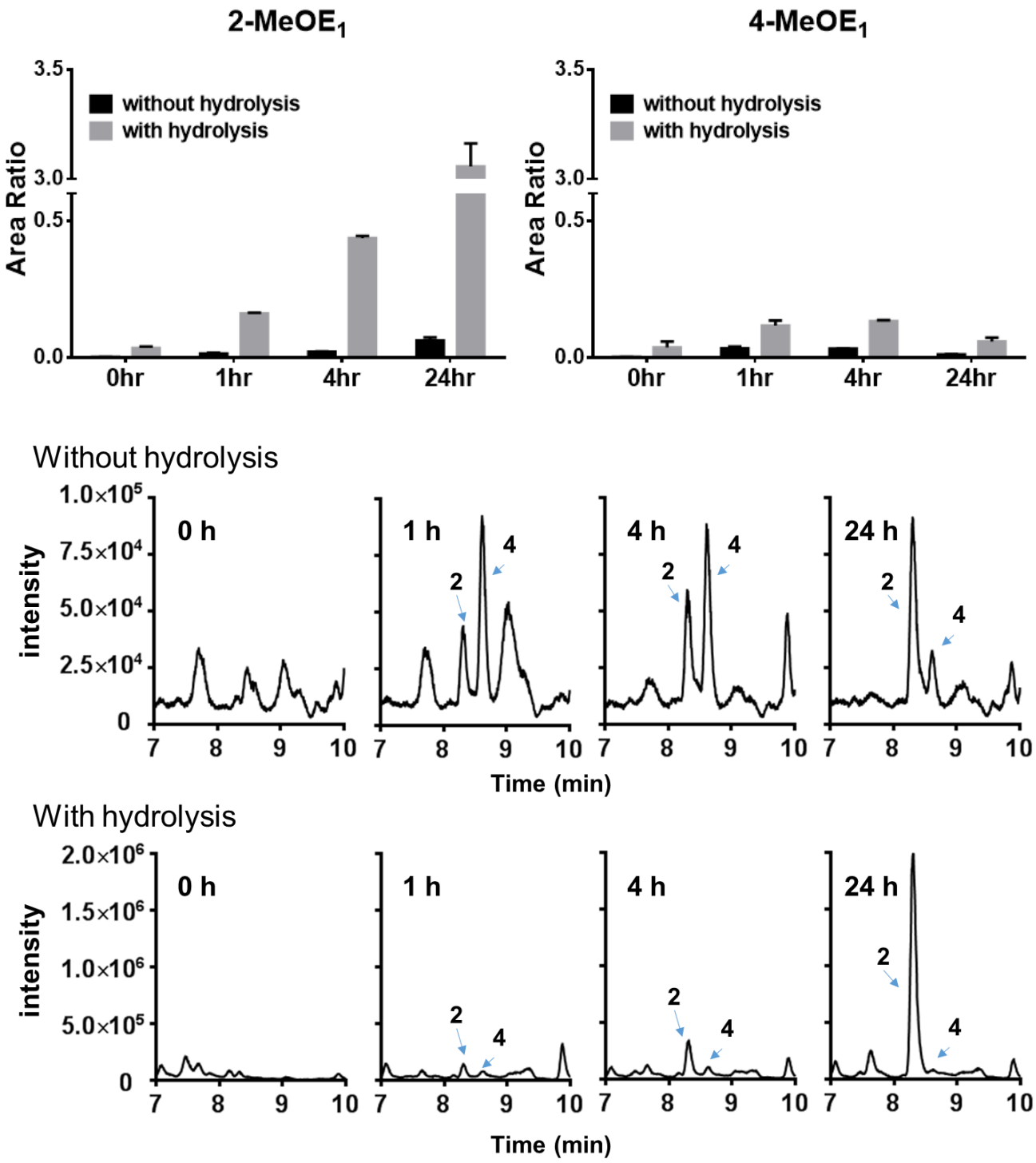


Figure 17. Time dependent formation of 2-MeOE$_1$ *in vivo*. ACI rats were dosed with estradiol-benzoate at 1 mg/kg subcutaneously in sesame oil and sacrificed at 0 h, 1 h, 4 h, and 24 h. Serum samples were prepared and analyzed using the LC-MS/MS method for 2- and 4-MeOE$_1$ before and after glucuronidase and sulfatase hydrolysis. Results were graphed and representative chromatograms were shown.

Since *in vivo* enzyme induction/modulation experiments typically last 3 to 7 days (Anadon et al., 2013; Sidorova et al., 2005), we chose to dose ACI rats daily with estradiol continuously for 4 days and measure the amount of 2-MeOE$_1$ 24 h after the last dosing. We further tested two different doses of estradiol-benzoate to find the dosing needed for the measurement of estrogen oxidative metabolism modulation. Groups of ACI rats were treated with vehicle, estradiol-benzoate at 0.1 mg/kg/day, or estradiol-benzoate 1 mg/kg/day for 4 days and analyzed for 2-MeOE$_1$. The results showed that 1 mg/kg/day dosing exhibited a good signal to noise ratio while 0.1 mg/kg/day dosing did not provide enough signal (Figure 18). Thus, estradiol benzoate at 1 mg/kg/day dosing was used for studies with botanical compounds and extracts.

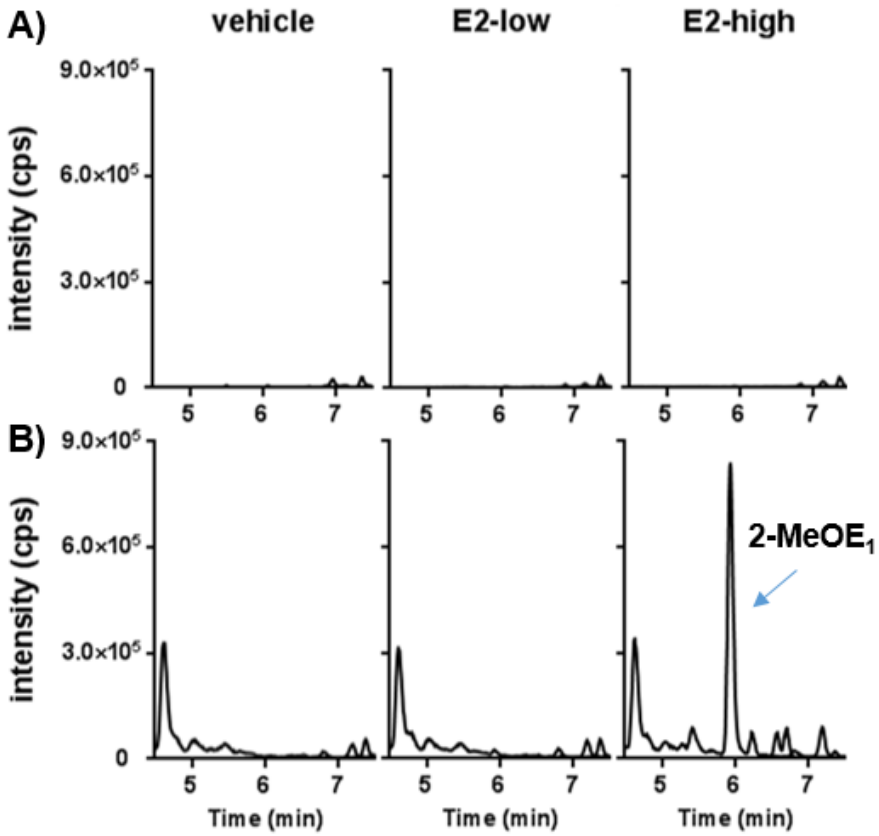


Figure 18. Representative chromatograms of serum methoxyestrones. Serum samples from vehicle, estradiol-benzoate 0.1 mg/kg/day (E2-low), and estradiol-benzoate 1 mg/kg/day (E2-high) treated ACI rats were analyzed for methoxyestrone metabolite A) without glucuronidase/sulfatase hydrolysis or B) after glucuronidase/sulfatase hydrolysis. Representative chromatograms were shown.

4. MODULATION OF ESTROGEN OXIDATIVE METABOLISM BY LICORICE SPECIES AND BIOACTIVE COMPOUNDS

(Reprinted in part with formatting changes from: Dunlap TL, Wang S, Simmler C, Chen SN, Pauli GF, Dietz BM, and Bolton JL. “Differential effects of glycyrrhiza species on genotoxic estrogen metabolism: licochalcone A downregulates P450 1B1, whereas isoliquiritigenin stimulates it” *Chemical Research in Toxicology* 28(8), 1584-1594 (2015))

4.1 Rationale and Hypothesis

Breast cancer is the second leading cause of cancer-related deaths among women in the United States (Siegel et al., 2014), and the risk of developing the disease increases with cumulative exposure to estrogens including hormone therapy (HT) (Cavalieri and Rogan, 2014; Siegel et al., 2014). The chemical mechanism of estrogen carcinogenesis involves metabolism of estrone/estradiol (E_1/E_2) to 2-hydroxy (non-toxic) and 4-hydroxy estrogen (genotoxic) metabolites by P450 1A1 and P450 1B1 enzymes, respectively (Figure 19). These estrogen metabolizing enzymes are typically up-regulated through activation of the AhR, which also controls polycyclic aromatic hydrocarbon (PAH) metabolism (Sorg, 2014). The genotoxic estrogen *o*-quinone formed from 4-hydroxy estrogen produces reactive oxygen species (ROS) and DNA adducts that induce malignant transformation of normal breast cells (Cavalieri and Rogan, 2010; Fernandez et al., 2006; Fernandez and Russo, 2010; Hemachandra et al., 2012; Russo et al., 2006; Yager, 2015). Alternatively, the estrogen *o*-quinones are reduced by *NAD(P)H:quinone oxidoreductase 1* (NQO1), which are up-regulated by antioxidant response element (ARE) activation (Figure 19) (Gaikwad et al., 2007).

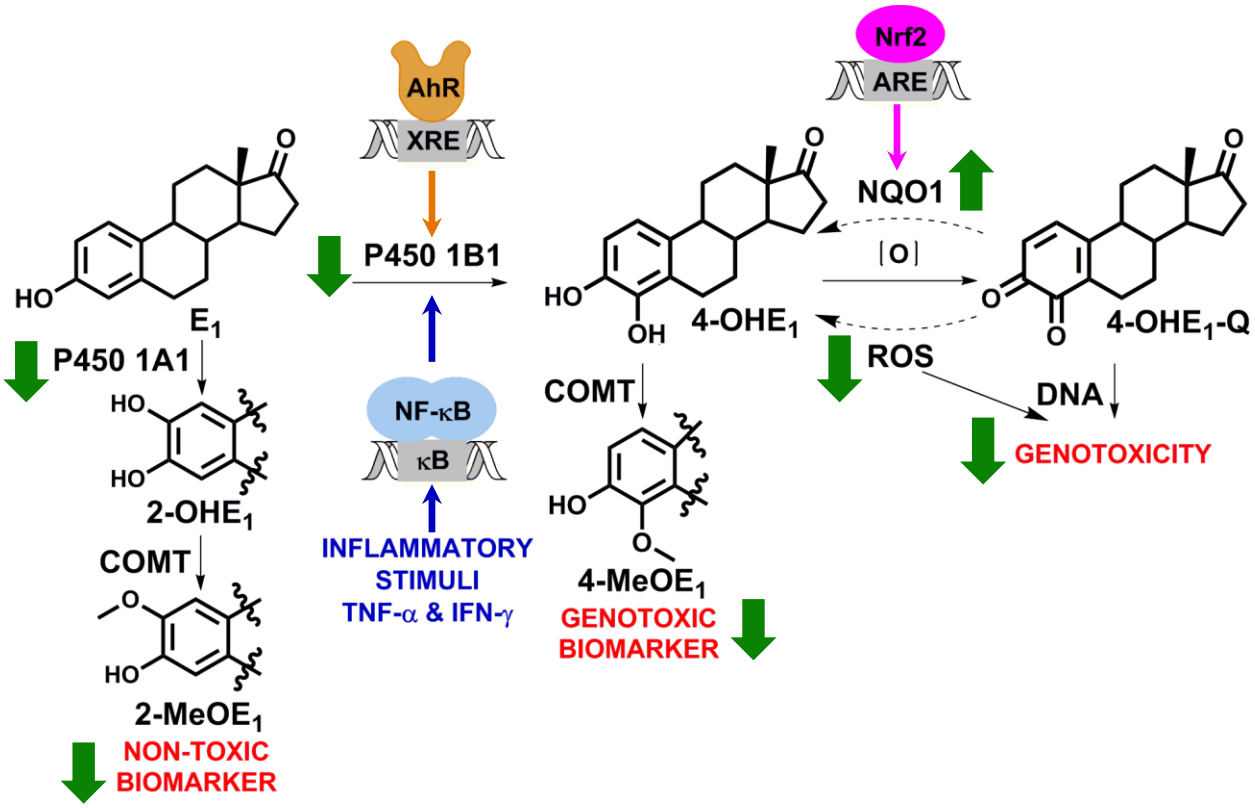


Figure 19. Hypothesis: effect of botanicals on inflammation-stimulated estrogen chemical carcinogenesis. Inflammation potentiates estrogen chemical carcinogenesis through activation of transcription factor NF-κB which up-regulates estrogen metabolizing enzymes P450 1A1 and 1B1 that are classically regulated by AhR (Figure has been simplified for clarity). P450 1B1 increases the estrogen metabolite 4-OHE1 which redox cycles with the genotoxic estrogen quinone 4-OHE1-Q to form ROS. E2 (not shown for clarity) is similarly metabolized to genotoxic metabolites. The hypothesis is that, to prevent estrogen carcinogenesis, several steps in the inflammation-stimulated estrogen carcinogenesis pathway could be modulated by chemopreventive botanicals, as indicated with green arrows.

It is becoming increasingly clear that inflammation plays a major role in breast cancer (Baumgarten and Frasor, 2012; Heinecke et al., 2014). However, the potential of inflammatory stimuli to enhance estrogen metabolism and increase breast cancer risk has unknown implications in women. Inflammation stimulates the NF-κB pathway, leading to

induction of the genotoxic estrogen 4-hydroxylase, P450 1B1 (Figure 19) (Patel et al., 2014; Šmerdová et al., 2014). P450 1B1 is also upregulated by prostaglandin E2 (PGE2) (Han et al., 2010) and inflammatory cytokines in breast and endometrial cancer cells (Han et al., 2010; Kamel et al., 2012; Salama et al., 2009). Furthermore, NF-κB can cooperatively bind with AhR to up-regulate xenobiotic response element (XRE)-responsive genes such as P450 1B1 (Vogel et al., 2014). As inflammation is often correlated with poor prognoses of both ER(-) and ER(+) breast cancers (Baumgarten and Frasor, 2012; Heinecke et al., 2014; Ristimaki et al., 2002), inflammation-stimulated estrogen carcinogenesis may take on a larger role in the initiation and progression of breast cancer than initially suspected.

Botanical dietary supplements are popularly used as natural alternatives to traditional HT and often contain a variety of chemopreventive polyphenols that have anti-inflammatory, antioxidant, chemopreventive, as well as weak estrogenic properties (Cuendet et al., 2010; Hajirahimkhan et al., 2013a; Hajirahimkhan et al., 2013b; Kim et al., 2008; Kühnl et al., 2015; Yao et al., 2015; Yu et al., 2014). The roots of licorice used medicinally according to worldwide pharmacopeias, including the United States Pharmacopeia and Chinese *Materia* Medica are *Glycyrrhiza glabra* (GG), *G. uralensis* (GU), and *G. inflata* (GI) (Kondo et al., 2007; Park et al., 2014) with GG being the most popular extract used in the United States (Hajirahimkhan et al., 2013a; Upton et al., 2010). Traditionally, licorice has been used to treat bacterial and viral infections, ulcers, inflammation/swelling, and asthma (Hajirahimkhan et al., 2013a; Kao et al., 2014). Today, licorice extracts are among some of the most popular botanicals for women's health in the United States (Hajirahimkhan et al., 2013a). As there is evidence that licorice extracts

contain compounds that have anti-inflammatory and chemopreventive properties (Cuendet et al., 2010; Kim et al., 2008; Kühnl et al., 2015), licorice may also have the potential to protect women from estrogen carcinogenesis (Figure 19).

All three *Glycyrrhiza* species contain the chalcone, isoliquiritigenin (LigC, C for chalcone) either as the aglycone or as its glycosides (Figure 6, Table I, Figure 8, 9), which induces detoxification enzymes including NQO1 (Cuendet et al., 2010; Cuendet et al., 2006; Gong et al., 2014). LigC is in equilibrium with the ERβ selective estrogenic flavanone, liquiritigenin (LigF, F for flavanone) (Hajirahimkhan et al., 2013a; Simmler et al., 2013b). GI also contains the Michael acceptor chalcone licochalcone A (LicA) as a major compound, which is not present in the other two species (Figure 6, Table I, Figure 8) (Farag et al., 2012). These differences in the chemical profiles (Table I, Figure 8) of medicinal licorice extracts suggest that their safety and efficacy in women will also be variable (Table I). The three medicinally used licorice species can be identified based on their aerial parts; however, the medical part of the plant, which is the root, has indistinguishable morphology which could lead to misidentification, and confusing biological data (Tao et al., 2013). In the current study, DNA fingerprinting and chemical profiling have been performed to unambiguously authenticate each species (Simmler et al., 2015). The effect of the individual *Glycyrrhiza* species (GG, GU, GI) and their constituents (LigF, LigC, LicA) on estrogen oxidative metabolism was then compared. The hypothesis was that these licorice polyphenols might modulate inflammation (i.e. iNOS activity) and P450 1B1 expression, through manipulation of NF-κB and AhR activation, and might decrease the formation of estrogen quinones (Figure 19). The data showed that the three *Glycyrrhiza* species contain different chemical profiles, which differentially modulate

estrogen metabolism further emphasizing the importance of standardization of botanical dietary supplements to species specific bioactive compounds.

4.2 Results

4.2.1. Bioactive compounds in *Glycyrrhiza* species.

In order to directly characterize and compare the most commonly used medicinal licorice species, we authenticated each species by DNA fingerprinting and chemical profiling (Simmler et al., 2015). Various glycosidated forms of LigF and LigC were present in the three *Glycyrrhiza* species (Figure 8 and 9). Their content was collectively expressed as % w/w of each extract as LigF equivalents (LigF, liquiritin, liquiritin apioside, and liquiritigenin 7-*O*-apiosylglucoside) and LigC equivalents (LigC, isoliquiritin, isoliquiritin apioside, and licuraside) (Table I, Figure 8, 9) (Simmler et al., 2015). Chalcone and flavanone glycosides can be considered as bio-precursors of the corresponding aglycones, LigF and LigC, since they are enzymatically deglycosylated by the gut flora *in vivo* (Asano et al., 2003; Kamei et al., 2005). LicA was only present in GI (5.42%), and not in glycosidated form. Due to the high content of LicA, the GI extract contained the highest total amount of chalcones (Table I), which are known for their anti-inflammatory and detoxification enzyme inducing properties (Chu et al., 2012a; Gong et al., 2014; Kühnl et al., 2015; Lau et al., 2010). In contrast, GU contained the lowest chalcone amount. These profiles showed that the three licorice extracts differed substantially in their composition of bioactive compounds.

Species	Compounds in licorice (% w/w)				
	LicA[a]	LigC	LigC equivalents[b]	LigF	LigF equivalents
GG	-	0.06 ± 0.00	2.97 ± 0.01	0.24 ± 0.01	5.61 ± 0.02
GU	-	0.09 ± 0.01	0.81 ± 0.03	0.41 ± 0.01	2.96 ± 0.03
GI	5.42 ± 0.34	0.13 ± 0.01	2.47 ± 0.05	0.12 ± 0.04	0.82 ± 0.06

Table I. Concentration of the bioactive markers in three licorice extracts determined by HPLC-UV. [a] LicA was below limit of detection in GG and GU. [b] The term LigC equivalents is used to represent the total amount of LigC aglycone plus LigC glycosides (isoliquiritin, isoliquiritin apioside, licuraside (Figure 9) in the crude extract. LigF equivalents is used to represent the total amount of LigF aglycone plus LigF glycosides (Liquiritin, liquiritin apioside, liquiritigenin -7-O-apiosylglucoside) in the crude extract. The values (% weight compound/weight crude extract) are expressed as mean ± SD from three independent measures.

4.2.2. Licorice extracts/compounds differentially modulated iNOS activity in macrophage cells.

Since inflammation can increase oxidative estrogen metabolism (Figure 19) (Salama et al., 2009), the anti-inflammatory activity of the licorice species and their major bioactive compounds was determined. Previous studies have measured the anti-inflammatory activities of some licorice species (Kim et al., 2006a; Park et al., 2014; Thiyagarajan et al., 2011; Wu et al., 2011); however, the current studies represent the first direct comparison of authenticated extracts and pure compounds. Among the three *Glycyrrhiza* species, GI was the most anti-inflammatory extract in RAW 264.7 macrophage cells, reducing iNOS activity to 35% of the LPS control versus 67% of control for GG at 20 μg/mL (Figure 20A). No anti-inflammatory effect was detected up to 20 μg/mL for GU. To determine the effect of the compounds on iNOS activity, LigF, LigC, and LicA were tested

in the Griess assay (Figure 20B). The chalcones, LicA and LigC, reduced iNOS activity in macrophage cells to 50% at 10 μM and to 25% and 20% (20 μM) of the LPS control, respectively. LigF did not reduce iNOS activity. These data showed that GI's anti-inflammatory activity is mainly due to LicA, which is only present in GI.

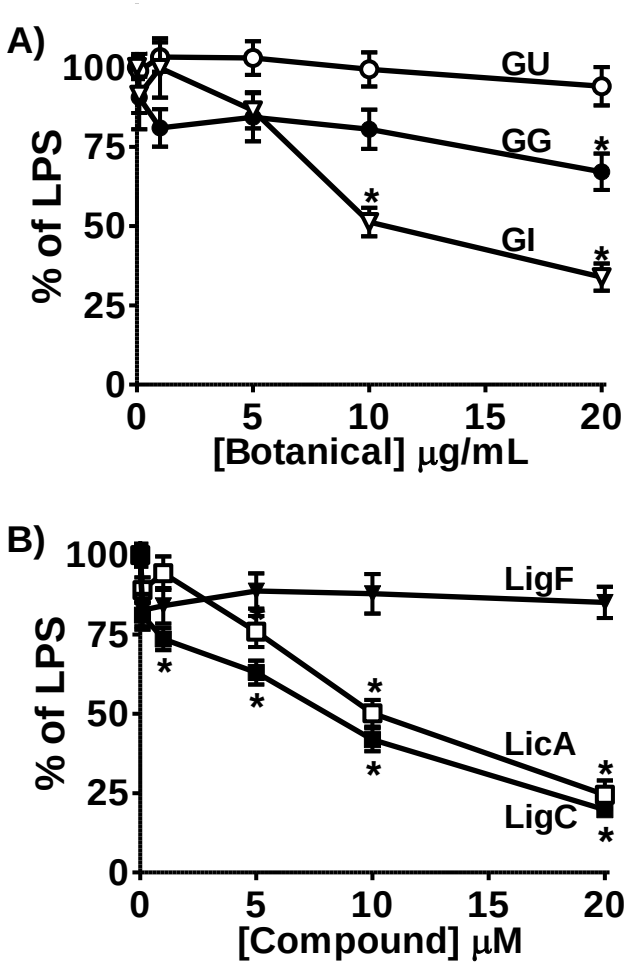


Figure 20. Licorice extracts/compounds differentially modulated iNOS activity in macrophage cells. Nitrite levels from macrophage RAW 264.7 cells were detected with Griess reagent after 24 h treatment with LPS (1 μg/mL) and A) GG, GU, and GI and B) licorice compounds LigF, LigC, and LicA. Results are the means ± SEM of three independent experiments analyzed by one-way ANOVA with Dunnett's multiple comparison post-test, *$p < 0.001$.

4.2.3. Licorice extracts/compounds differentially modulated estrogen metabolism.

The improved LC-MS/MS assay was utilized to determine if the *Glycyrrhiza* species and their compounds alter estrogen oxidative metabolism. The amount of 2- and 4-$MeOE_1$ produced in the control cells treated with E_2 was set to 100%. GI (5 μg/mL) was the only *Glycyrrhiza* species tested which inhibited the formation of 2-$MeOE_1$ and 4-$MeOE_1$ metabolites in MCF-10A cells (Figure 21A, B). In contrast, both GG and GU increased 4-$MeOE_1$ to 150% of control. Experiments with LigC showed that it significantly increased both 2-$MeOE_1$ and 4-$MeOE_1$ at 1 μM and gave a maximum enhancement up to 300% of control at 10 μM (Figure 21C, D). In contrast, LicA dose-dependently reduced both 2-$MeOE_1$ and 4-$MeOE_1$ to 37% and 12% of control, respectively, at 10 μM (Figure 21C, D). LigF very moderately increased both metabolites at 10 μM likely due to equilibration with its chalcone isomer LigC (Simmler et al., 2013a). These data showed that the three medicinal licorice extracts and their bioactive compounds have differential effects on estrogen metabolism.

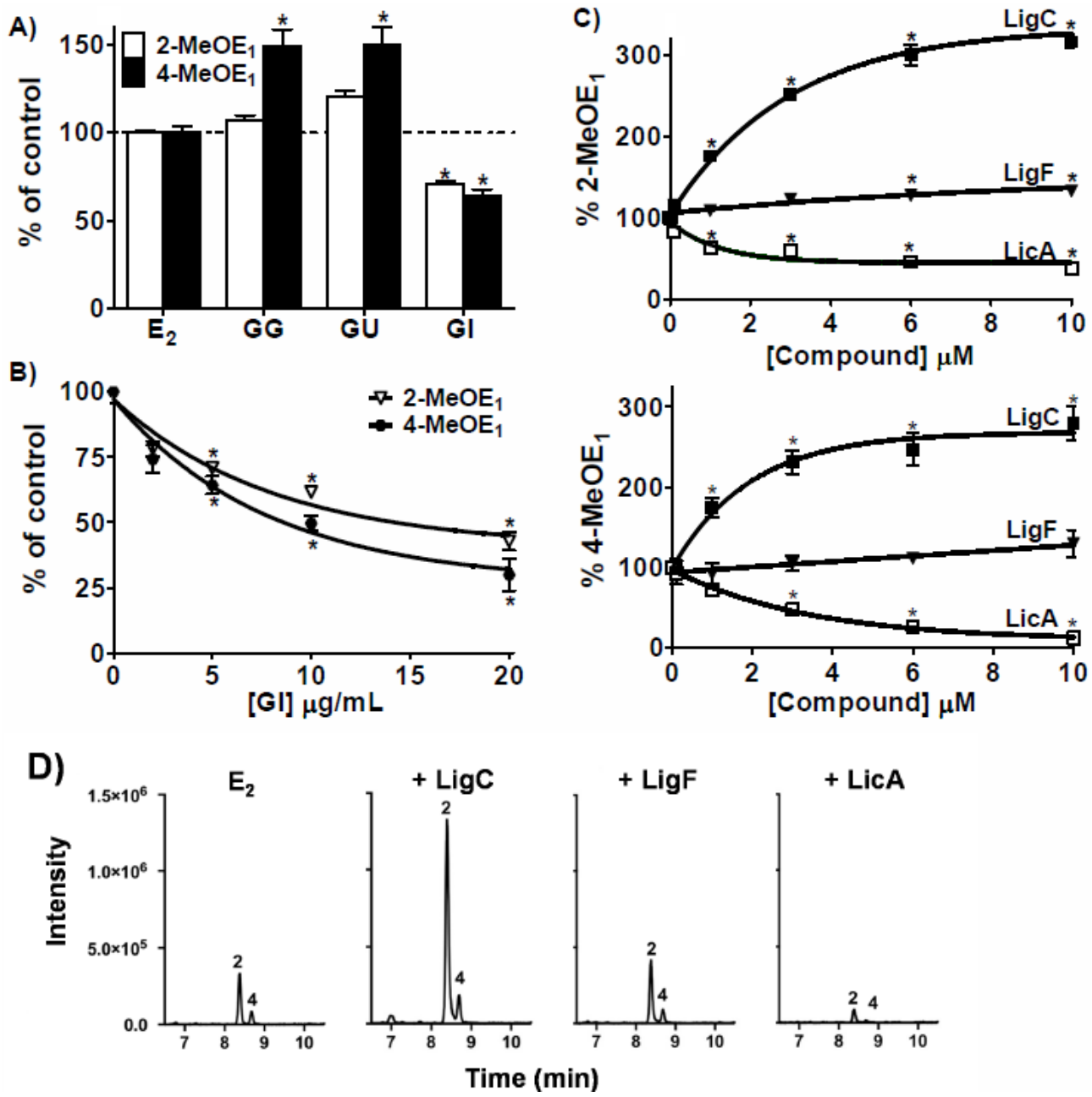


Figure 21. Licorice extracts/compounds differentially modulated estrogen metabolism. MCF-10A cells treated for 3 days with E_2 (1 µM) and A) GG, GU, and GI (5 µg/mL) or B) various doses of GI. C) After 3 days treatment of MCF-10A cells with E_2 (1 µM) and LigF, LigC, and LicA, media were collected and analyzed for 2-$MeOE_1$ and 4-$MeOE_1$ metabolites by LC-MS/MS. Results are the means ± SEM of three independent experiments analyzed by one-way ANOVA with Dunnett's multiple comparison post-test, *$p < 0.001$. D) Representative SRM chromatograms with transition 534.4 – 171.2 showing 2-$MeOE_1$ and 4-$MeOE_1$ peaks after 3 days treatment of MCF-10A cells with E_2 (1 µM) and LigF, LigC, and LicA (10 µM). D) Representative SRM chromatograms with transition 534.4 – 171.2 showing 2-MeOE1 and 4-MeOE1 peaks after 3 days treatment of MCF-10A cells with E2 (1 µM) and LigF, LigC, and LicA (10 µM).

4.2.4. Licorice extracts/compounds differentially modulated inflammation-stimulated P450 1B1 gene expression in MCF-10A cells.

It has been shown that the inflammatory cytokine TNF-α induces P450 1B1 gene expression in breast cancer cell lines (MCF-7) (Salama et al., 2009). Similar experiments were performed using non-tumorigenic breast epithelial cells (MCF-10A). Treatment with TNF-α alone had little effect on P450 1B1, whereas co-treatment with TNF-α and IFN-γ elicited a 3-fold induction (Figure 22A). To determine if AhR or NF-κB inhibition could reduce cytokine-stimulated P450 1B1 mRNA, cells were treated with a combination of cytokines (TNF-α and IFN-γ) and AhR antagonist (CH-223191) (Kim et al., 2006b) or NF-κB pathway inhibitor (IKK inhibitor VII) (Waelchli et al., 2006). Both inhibited cytokine-stimulated P450 1B1 to nearly the levels of the DMSO control. These data extended literature reports (Kamel et al., 2012; Salama et al., 2009; Šmerdová et al., 2014; Umannová et al., 2008) by showing that inflammatory cytokines could induce P450 1B1 in "normal" breast epithelial cells (MCF-10A) as well as in cancer cells.

To determine the effect of licorice on inflammation-stimulated P450 1B1 mRNA expression in MCF-10A cells, extracts of GG, GU, and GI (5 μg/mL) were incubated with and without cytokines for 24 h (Figure 22B). Both GU and GG increased P450 1B1 mRNA expression, particularly in the presence of cytokines. In contrast, GI treatment led to a 10-fold reduction with or without cytokines. To explain this difference in activity between *Glycyrrhiza* species, LigF, LigC, and LicA were also evaluated (Figure 22C). LicA (1 μM) strongly inhibited P450 1B1 mRNA expression to 20% of DMSO control and 5% of cytokine controls (Figure 22C). LicA also dose-dependently reduced basal and cytokine-induced P450 1B1 mRNA levels (Figure 22D) significantly at 0.5 μM. In contrast, LigC (1

μM) increased P450 1B1 mRNA expression to approximately 200% of the DMSO control and potentiated cytokine-induced P450 1B1 mRNA expression by an additional 2-fold (Figure 22C). LigF did not affect P450 1B1 mRNA expression. These data showed that LicA and LigC and the three licorice species have differential effects on P450 1B1 expression, which mirror estrogen metabolism data.

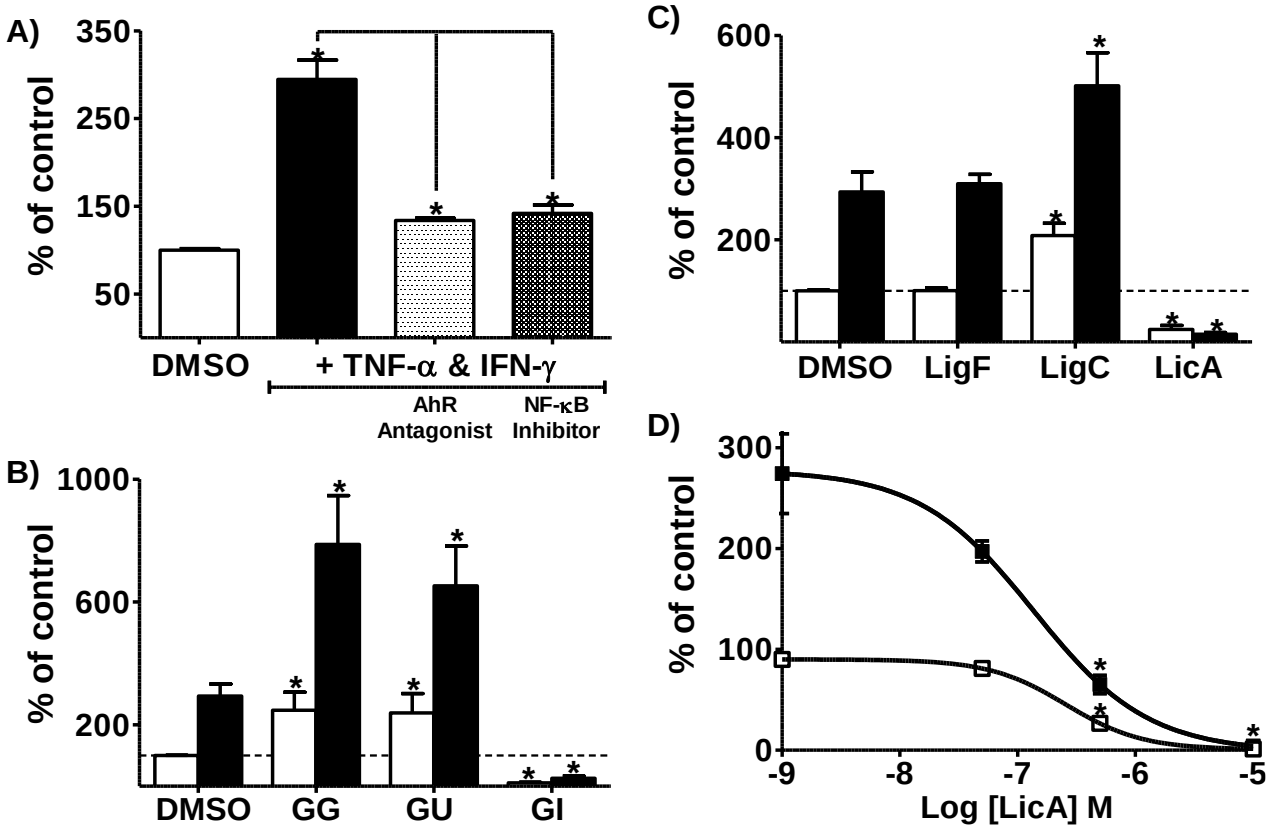


Figure 22. Licorice extracts/compounds differentially modulated inflammation-stimulated P450 1B1 gene expression in MCF-10A cells. P450 1B1 mRNA expression analyzed after 24 h via qPCR in MCF-10A cells with cytokines (TNF-α and IFN-γ, 10 ng/mL each) (black bars) and without cytokines (open bars). A) AhR antagonist CH-223191 (100 nM) and NF-κB pathway inhibitor IKK inhibitor VII (2 μM). MCF-10A cells treated with B) GG, GU, and GI (5 μg/mL) C) LigF, LigC, and LicA (1 μM). Results with extracts/compounds were analyzed by Student's t-test, *$p < 0.05$ (GG, GU, LigC) and *$p < 0.001$ (GI and LicA) to compare treatment groups to DMSO and cytokine controls. D) Dose-response for LicA (open squares) and LicA + TNF-α and IFN-γ (black squares). Results are the means ± SEM of three independent experiments analyzed by one-way ANOVA with Dunnett's multiple comparison post-test, *$p < 0.001$.

4.2.5. Licorice compounds modulated TCDD-induced P450 1B1 gene expression, P450 1A/1B activity, and XRE-luciferase reporter activity.

In order to determine the mechanism of licorice compound modulation of P450 1B1, MCF-10A cells were treated with the AhR agonist 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Denison and Nagy, 2003; Murray et al., 2014). TCDD strongly increased P450 1B1 induction 20-fold. LigC slightly increased TCDD-induced P450 1B1 mRNA expression, whereas LigF had no effect (Figure 23A). In contrast, LicA (10 μM) treatment dramatically decreased TCDD-induced P450 1B1 mRNA expression to 10% of the TCDD response (Figure 23A). Since licorice compounds modulated estrogen metabolism and P450 1B1 expression, P450 1A/1B activity was also determined in MCF-10A cells after treatment of cells with DMSO or TCDD and compounds for 48 h (Figure 23B). TCDD induced P450 1A/1B activity 30-fold. LicA (10 μM) completely inhibited both P450 1A/1B activities of DMSO and TCDD controls. In contrast, LigC (10 μM) enhanced P450 1A/1B activity to more than 5-fold and also increased TCDD-induced P450 1A/1B activity significantly. LigF had no effect on P450 1A/1B activity. These results mirrored the observations made with estrogen metabolism and P450 mRNA expression, in which LicA inhibited, LigC stimulated, and LigF had little effect.

To investigate the mechanism by which LicA inhibited P450 1B1 gene expression and P450 1A/1B activities, XRE-luciferase reporter activity was analyzed in HepG2 cells. LicA dose-dependently inhibited TCDD-induced XRE-luciferase reporter activity showing significant inhibition (Figure 23C, IC_{50} = 12.3 μM). While LigC slightly increased XRE-luciferase reporter activity, LigF had no effect (Figure 24). These data suggest that LicA can antagonize AhR, resulting in downregulation of P450 1A/1B, leading to inhibition of estrogen metabolism. In contrast, LigC may only be a very weak AhR agonist and likely has other biological targets, which induce P450 1B1 expression and P450 1A/1B activity.

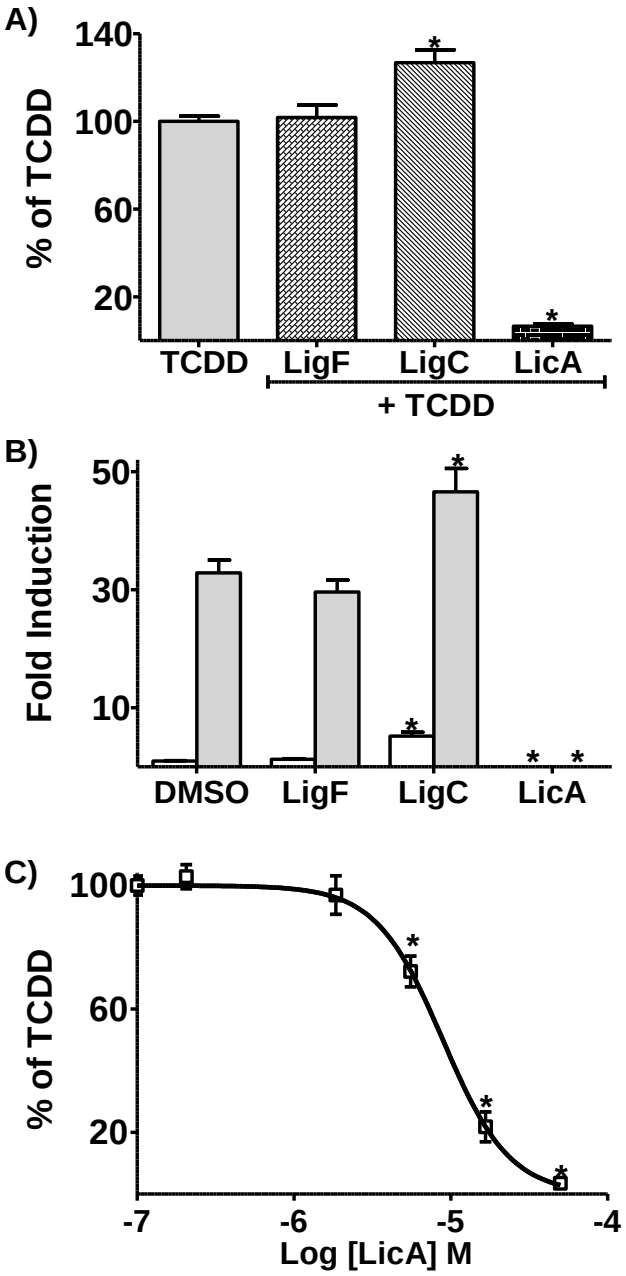


Figure 23. LicA down-regulated TCDD-induced P450 1B1 mRNA expression, P450 1A/1B activity, and XRE-luciferase activity. A) P450 1B1 mRNA expression was analyzed via qPCR after 24 h treatment of MCF-10A cells with TCDD (10 nM) and licorice compounds (10 μM). B) P450 1A/1B activity measured via EROD assay in MCF-10A cells after treatment with compounds (10 μM) 1 h prior to treatment with TCDD (10 nM) for an additional 48 h. C) HepG2 cells were incubated with TCDD (10 nM) and LicA for 24 h before analysis of XRE-luciferase reporter activity. Results are the means ± SEM of three independent experiments analyzed by one-way ANOVA with Dunnett's multiple comparison post-test, *$p < 0.001$.

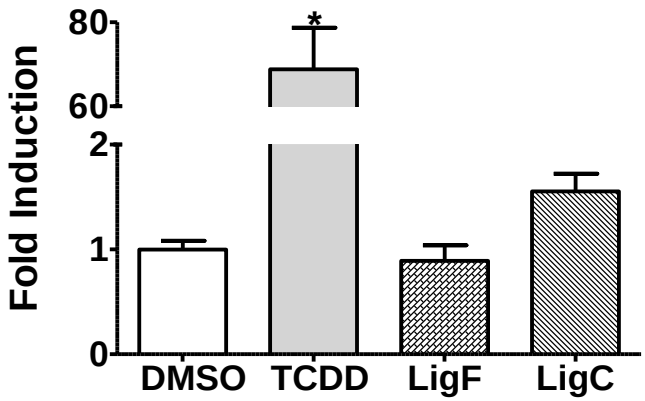


Figure 24. Effect of LigC and LigF on XRE-luciferase activity. HepG2 cells were incubated with TCDD (10 nM) or TCDD with LigF/LigC (10 µM) for 24 h before analysis of XRE-luciferase reporter activity. Results are means ± SEM analyzed by one-way ANOVA with Dunnett's multiple comparison post-test, *p < 0.001.

LicA contains a Michael acceptor, and has been shown previously to covalently react with IKK through the Michael acceptor to exert anti-inflammatory activity (Funakoshi-Tago et al., 2009). When the Michael acceptor was replaced with saturated hydrocarbons, the compound displayed no anti-inflammatory activity (Funakoshi-Tago et al., 2010a). which could contribute to AhR inhibition through covalent binding to nucleophilic amino acide residues in the AhR binding pocket. In the binding pocket of AhR, two nucleophilic amino acid residues were found to be important for its function. His291 has been mostly studied and found to be important for maintaining ligand binding activity; Cys333 has also been shown to help maintain normal binding function of AhR with free thiol sticking out in the binding pocket (Bisson et al., 2009; Pandini et al., 2009). To determine if LicA antagonizes AhR through covalent binding with the key amino acids in the binding pocket, recombinant human AhR (10 µM) was incubated with and without LicA (10 µM) for 1 h at

37 °C in PBS. The samples were then processed and analyzed using AutoFlex Speed LRF MALDI-TOF mass spectrometer (Figure 25). However, the results did not show the covalent binding of LicA with AhR, which indicates the inhibition of AhR could be mediated by simple receptor occupation, or interaction with upstream targets. Western blot and immunohistochemistry analysis of AhR could be conducted for more mechanistic information regarding the inhibitory effects of LicA.

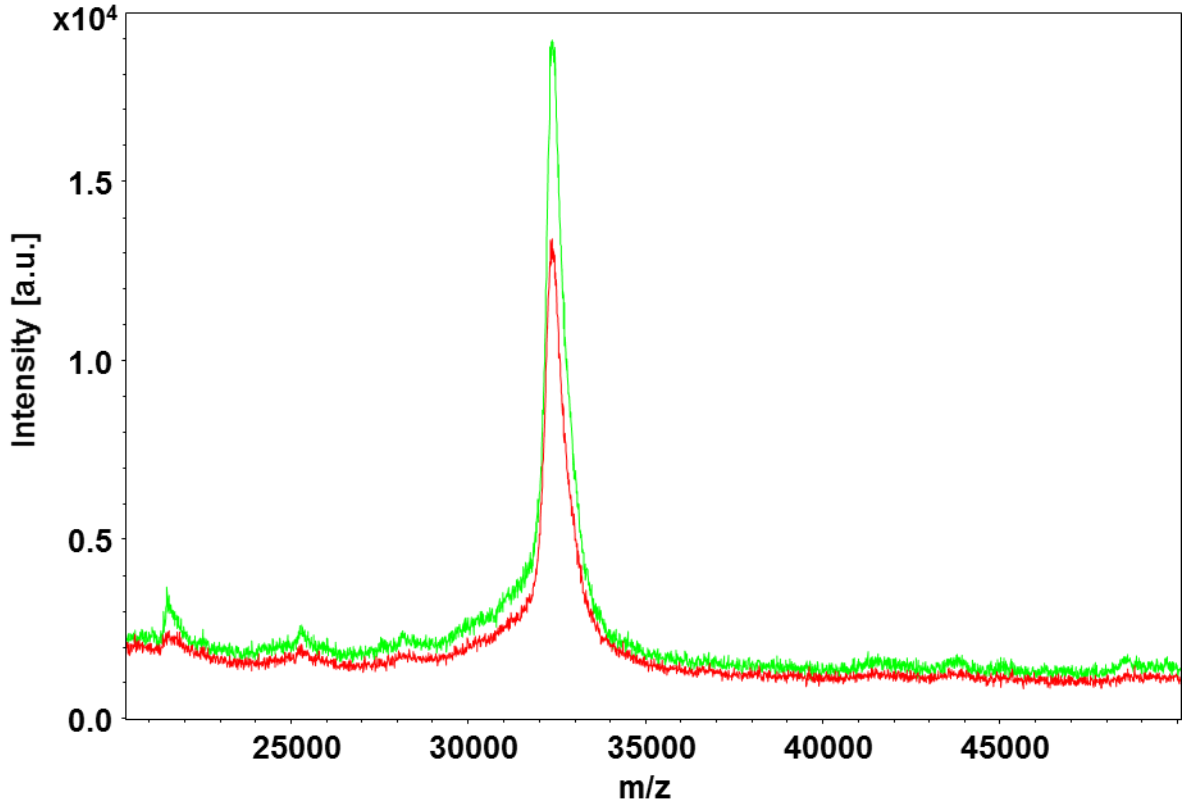


Figure 25. MALDI mass spectra of AhR and LicA incubations. Human recombinant AhR protein (10 µM) were mixed with LicA (10 µM) or blank control and incubated at 37°C for 1 hr. The samples were then prepared and analyzed by MALDI-TOF. The mass spectrums of the two incubations were shown (green: AhR by itself; red: AhR and LicA).

4.2.6. LicA only moderately inhibited P450 1A/1B activity in MCF-10A cells and did not show P450 1A1 or 1B1 selectivity.

It is possible that the modulatory effects observed above could be due to direct effects on P450 1A/1B activity. We performed inhibition assays using recombinant proteins as well as testing the overall P450 1A/1B inhibitory effect in MCF-10A cells. After induction of P450 1A/1B with TCDD, cells were treated with compounds and substrate (EROD) for 20 min, to directly determine the effect of the compounds on P450 1A/1B enzyme activity. No inhibition of P450 1A/1B activity was observed in the MCF-10A cells except at the highest concentration of LicA (10 µM, Figure 26A). Inhibitory effects were only observed in experiments with the purified enzymes at the highest concentrations of chalcones (10 µM) and no selectivity of P450 1A/1B enzyme activity was detected (Figure 26B, C). These data suggest that modulation of P450 protein levels by compounds in licorice extracts rather than direct inhibitory effects are most likely responsible for changes in estrogen metabolism.

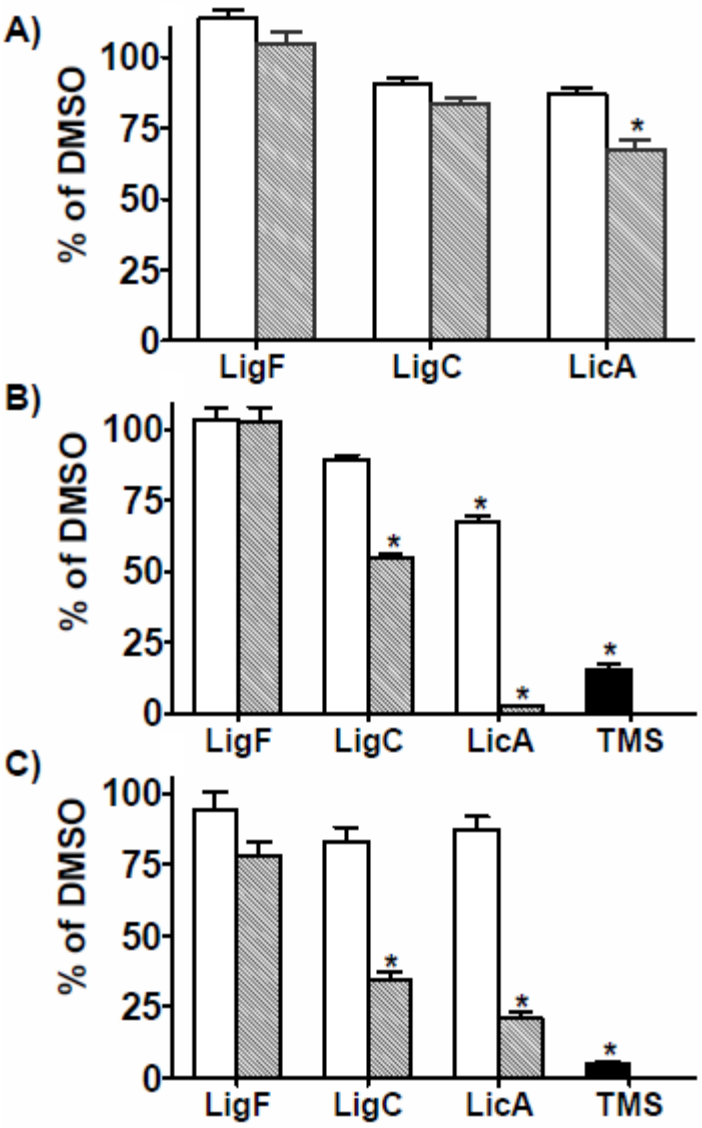


Figure 26. LicA only moderately inhibited P450 1A/1B activity in MCF-10A cells and did not show P450 1A1 or 1B1 inhibition selectivity. A) MCF-10A cells were pretreated with TCDD (10 nM) for 48 h to induce P450 enzymes and thereafter pre-incubated with LigF/LigC/LicA (1 µM, open bars) and (10 µM, striped bars) for 5 min at 37°C before ethoxyresorufin and NADPH was added for 20 min. B) Human recombinant P450 1A1 and C) P450 1B1 protein with reductase were incubated with ethoxyresorufin, NADPH and LigF/LigC/LicA (1 µM, open bars) and (10 µM, striped bars) or 2,3',4,5'-tetramethoxystilbene (TMS) (2 µM) at 37°C for 20 min. EROD activity was measured and results were analyzed by one-way ANOVA with Dunnett's multiple comparison post-test, * $p < 0.001$.

4.2.7. LicA inhibited estrogen oxidative metabolism and induced NQO1 activity *in vivo.*

ACI rat is a rat model frequently used in estrogen carcinogenesis studies because of its high sensitivity to develop mammary tumors upon estrogen treatment (Shull et al., 1997). In this study, LicA was further tested in this rat model for its ability to modulate estrogen metabolism. However, rat P450 1B1 has been shown to preferentially catalyze estrogen 2-hydroxylation and lack 4-hydroxylation capability due to species differences (Nishida et al., 2013). Thus, the 2-methoxyestrone metabolite was used as a marker for the measurement of overall estrogen metabolism.

To avoid extensive first-pass effect, which is common for polyphenolic flavonoids and chalcones, LicA was administered subcutaneously together with estradiol-benzoate (1 mg/kg/day) for 4 days. At 80 mg/kg/day, LicA was able to significantly reduce the amount of 2-MeOE$_1$ as compared to the estradiol-benzoate only group (Figure 27). Additionally, since LicA was previously shown to activate Nrf2 and have NQO1 induction activity, the NQO1 activity was measured with liver samples collected from the rats (Hajirahimkhan et al., 2015; Kühnl et al., 2015). The results showed that LicA also moderately induced NQO1 activity in liver, which is statistically significant compared to control samples (Figure 28). Analysis of the concentration of free LicA in serum and liver showed that serum concentration of LicA was 259 ± 44 nM in the LicA dosed group and not detected in other groups (Table II). Liver concentration of LicA was 66 ± 16 ng/g in the LicA dosed group, which is comparable to the serum level.

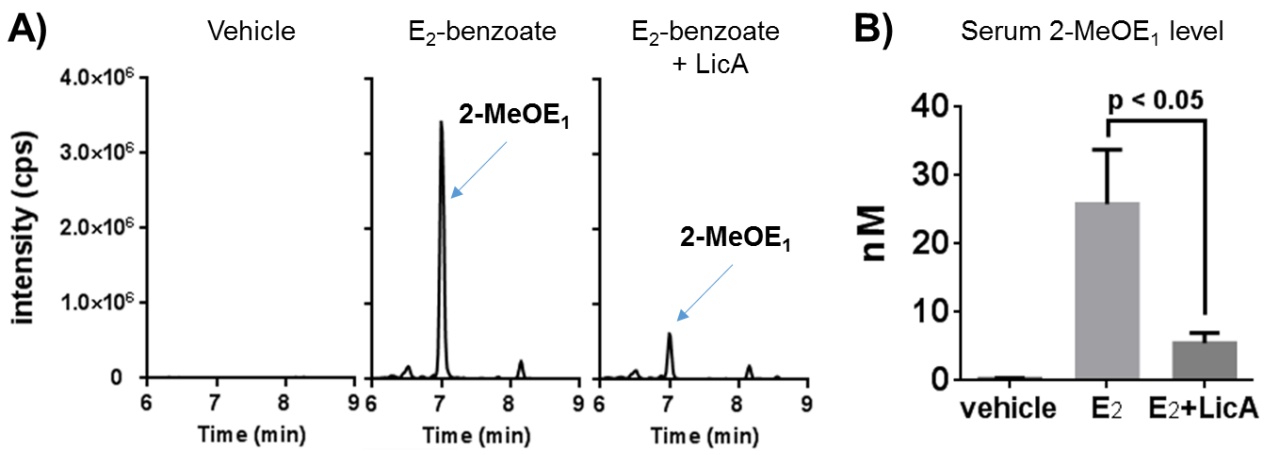


Figure 27. LicA significantly reduced serum 2-MeOE$_1$ in ACI rats. Female ACI rats at 6 weeks of age were administered subcutaneously daily for 4 days with vehicle (sesame oil), estradiol-benzoate (1 mg/kg/day), and estradiol-benzoate (1 mg/kg/day) plus LicA (80 mg/kg/day). Serum 2-MeOE$_1$ were measured after glucuronidase and sulfatase hydrolysis. A) Representative chromatograms were shown. B) Quantitative results were plotted as the average with error of 6 animals per group and data was analyzed by student t-test.

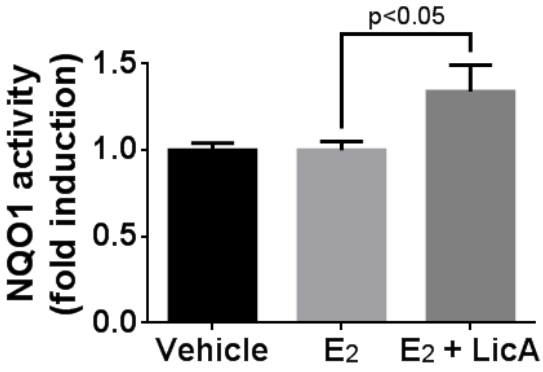


Figure 28. LicA induced liver NQO1 activity in ACI rats. Liver tissues were collected from female ACI rats treated with vehicle, E$_2$-benzoate (1 mg/kg/day), and E$_2$-benzoate (1 mg/kg/day) + LicA (80 mg/kg/day) for 4 days. Tissue homogenates were analyzed for their NQO1 activity with the NQO1 activity assay using menadione and MTT. Six samples per treatment group were plotted as mean ± standard error and analyzed by student t-test. (Data generated by Huali Dong)

Rat number	Serum (nM)	Liver (ng/g)	Mammary (ng/g)
1	381.58 ± 8.74	108.72 ± 4.01	24.30 ± 2.07
2	208.69 ± 15.03	42.73 ± 2.92	10.14 ± 0.56
3	112.13 ± 5.47	8.64 ± 1.07	11.33 ± 0.51
4	390.75 ± 6.32	88.45 ± 2.49	19.89 ± 0.70
5	206.50 ± 2.31	46.57 ± 1.82	15.61 ± 1.03
6	251.43 ± 7.67	93.33 ± 2.41	30.42 ± 1.56

Table II. Concentrations of LicA in serum, liver, and mammary samples determined by UHPLC-MS/MS. Serum, liver, and mammary samples from the LicA treatment group were analyzed for free LicA concentration. LicA concentrations were expressed as mean ± SD from five analytical replicates. (Data generated by Lingyi Huang)

As the first study that measured estrogen oxidative metabolism modulation *in vivo*, results from this study have confirmed the activity of LicA determined *in vitro* and further provided data for the use of ACI rat model for future estrogen oxidative metabolism modulation studies. However, since the catalytic activity of rat P450 1B1 is completely opposite to that of human P450 1B1 in regards of estrogen 2- and 4-hydroxylation metabolism, specificity on P450 1A1 or P450 1B1 could not be interpreted by using 2-$MeOE_1$ as the marker. Other biological readout such as protein or mRNA could be measured to help elucidate the data. Humanized mice could also be used as a tool to study the effect. However, since the local production and metabolism of estradiol plays a key role in estrogen chemical carcinogenesis, P450 1A1 and 1B1 gene modified mice/rats with human sequence could be used as a better way to mimic human estrogen oxidative metabolism. Bioactive compounds identified from *in vitro* studies sometimes do not carry over to the *in vivo* system due to the differences between single cell type *in vitro* and

various cell types in tissues and organs *in vivo*. These differences also affected the local concentration of the compound of interest. Previously, LicA has shown potent anti-inflammatory activity *in vivo* at 80 mg/kg dosing through i.p. injection with no observed toxicity (Chu et al., 2012b). Since LicA had μM anti-inflammatory activity *in vitro*, the compound exposure at 80 mg/kg was enough to have the corresponding biological activity. Our results on the estrogen metabolism modulation also showed low μM activity of LicA (Figure 21). Thus in the current study, to maximize compound exposure and avoid toxicity, LicA was dosed at 80 mg/kg/day through co-injection with estradiol-benzoate subcutaneously. The serum concentration of LicA was determined to be around 250 nM 24 h after the last dosing, however, a systematic multi-point PK study would be needed for the estimation of the real compound exposure.

In conclusion, LicA displayed significant effects in down-regulation of estrogen oxidative metabolism and showed activity in both *in vitro* and *in vivo* systems. Future studies can also be conducted with the glucuronidase and sulfatase hydrolysis procedure incorporated to the *in vitro* studies to be able to use lower estradiol treatment concentration and better correlate the results *in vivo*. Further studies will be needed with oral administration of the licorice extracts to determine if the extracts that contain LicA exert similar activity after first-pass metabolism with the presence of various other bioactive compounds.

4.3. Discussion

The Women's Health Initiative has shown that traditional HT can increase breast cancer risk. Estrogens are metabolized by P450 1A1/1B1 to 2- and 4-hydroxy metabolites,

respectively. The 4-hydroxy metabolite forms genotoxic quinones that cause depurinating, mutagenic DNA adducts. P450 1A1 2-hydroxylation generates quinones that form stable adducts, which are not tumorigenic (Cavalieri et al., 1997). It is also known that inflammation could enhance chemical estrogen carcinogenesis by up-regulating P450 1B1 (Figure 19) (Han et al., 2010; Kamel et al., 2012; Patel et al., 2014; Salama et al., 2009; Šmerdová et al., 2014; Umannová et al., 2008), while down-regulating P450 1A1 (Šmerdová et al., 2014). In this study, cytokines up-regulated P450 1B1 in non-tumorigenic breast epithelial cells (MCF-10A), similar to what was observed in MCF-7 breast cancer cells (Kamel et al., 2012). When either AhR or NF-κB activation was inhibited with an antagonist and IKK inhibitor, cytokine-induced P450 1B1 expression was reduced to nearly DMSO control levels (Figure 22A). These data suggested that botanicals that target both NF-κB and AhR might inhibit estrogen oxidative metabolism.

Licorice is currently used in many women's botanical dietary supplements because of its estrogenic properties, likely due to the weak ER ligand, LigF (Hajirahimkhan et al., 2013a; Snelten et al., 2012). Only three licorice species, GG, GU, and GI, are approved for use in licorice botanical dietary supplements, which usually consist of GG in the United States (Hajirahimkhan et al., 2013a). The roots of the three licorice species have indistinguishable morphology and could be misidentified (Tao et al., 2013). Thus, DNA fingerprinting and chemical profiling must be performed to authenticate each species. In this study, the three Pharmacopeia licorice species have been carefully authenticated and chemically profiled and systematically analyzed for their effect on inflammation and estrogen metabolism.

GG, GU, and GI showed moderate estrogenic activity with EC_{50} around 10 µg/ml in induction of alkaline phosphatase and significantly induced ERE activation at 10 µg/ml (Hajirahimkhan et al., 2013b). LigC and LigF showed comparable estrogenic activity in alkaline phosphatase induction, but LigF displayed selective binding to ERβ while LigC bound to both ERα and ERβ. The anti-inflammatory activities between these three *Glycyrrhiza* species were highly dependent on the amount of chalcones. GI was the most anti-inflammatory botanical compared to GU and GG (Figure 20A), probably because of the high quantity of the Michael acceptor chalcone, LicA (Figure 20B). In support of the importance of the α,β-unsaturated carbonyl in LicA, it has been shown that reducing the double bond eliminated its anti-inflammatory effects (Funakoshi-Tago et al., 2010b). GG and GU share similar chemical profile, but GG has more LigC and LigF equivalents as well as isoliquiritin equivalents (Table I). Glabridin was also uniquely found in GG. GG also showed anti-inflammatory activity likely due to LigC. GU showed no anti-inflammatory effect, since the concentrations of LigC was much lower than in GG. Both LicA and LigC Michael acceptors could potentially modify cysteine residues on NF-κB covalently, leading to inhibition of NF-κB DNA binding and in turn iNOS expression/activity, similar to other alkylating agents that modify p50 or p65 NF-κB subunits (Linnewiel-Hermoni et al., 2014; Switzer et al., 2012; Xia et al., 2004). Alternativley, it has been reported that LigC might demonstrate anti-inflammatory activity through suppression of IKK, ERK1/2 and p38 phosphorylation (Kim et al., 2008). The flavanone LigF, which lacks the Michael acceptor moiety, did not have anti-inflammatory activity. These results were similar to what has been reported in other anti-inflammatory studies which showed that LigC >> LigF (Feldman et al., 2011; Honda et al., 2012). Anti-inflammatory activities have also been

shown for different extracts of GG (Park et al., 2014; Thiyagarajan et al., 2011), GU (Park et al., 2014; Wu et al., 2011), and GI (Kim et al., 2006a). However, various experimental conditions were used making comparisons between the anti-inflammatory activities of the licorice species difficult. The present study gives a direct comparison of authenticated and chemically profiled licorice species and their bioactive compounds on reducing inflammation in a cell based system.

The LC-MS/MS methods previously described (Hemachandra et al., 2012; Xu et al., 2007a) were optimized to monitor the effect of licorice extracts/compounds on P450 mediated estrogen metabolism by measuring 2-$MeOE_1$ (non-toxic biomarker) and 4-$MeOE_1$ (genotoxic biomarker) in MCF-10A cells. Only GI inhibited estrogen metabolism (Figure 21), as well as reducing basal and cytokine-induced P450 1B1 mRNA levels (Figure 22). LicA, the major compound in GI (Table I), was also the only compound that inhibited estrogen metabolism (Figure 21C, D), P450 1B1 mRNA levels (Figure 22C, D, 15A), TCDD-induced P450 1A/1B activity (Figure 23B), and TCDD-induced XRE-luciferase reporter activity (Figure 23C), all at low μM concentrations. Collectively, these data suggested that LicA modulates estrogen metabolism through antagonizing AhR. However, since it is possible that LicA might directly inhibit P450 1A1/1B1 activity, inhibition assays were carried out with MCF-10A cells and recombinant proteins. While LicA and LigC led to a significant inhibition of recombinant P450 1A1/1B1 enzyme activity (Figure 26B, C), LicA (10 μM) only inhibited 25% of P450 1A/1B enzyme activity in MCF-10A cells (Figure 26A). No selective inhibition of P450 1A1 or 1B1 was detected. These data suggest that regulatory suppression of P450 1B1 mRNA/protein levels by LicA (0.5 μM, Figure 22D)

rather than inhibition of enzyme activities (10 μM, Figure 26A) dominates its inhibitory effect on 2- and 4-$MeOE_1$ formation.

Other natural products have been shown to inhibit estrogen oxidative metabolism. For instance, the chemopreventive compound, resveratrol, reduces estrogen oxidative metabolism (Chen et al., 2004; Lu et al., 2008). Resveratrol (20-25 μM) reduced TCDD-induced P450 1B1 expression, decreased oxidative estrogen metabolism, and DNA damage (Chen et al., 2004; Lu et al., 2008), resulting in reduced E_2/TCDD-induced malignant transformation in MCF-10F cells (Lu et al., 2008). Resveratrol reduced P450 1A1 or 1B1 expression in several cell lines and decreased TCDD-induced AhR binding (Beedanagari et al., 2009; Chen et al., 2004; Ciolino and Yeh, 1999), suggesting that resveratrol is a natural AhR antagonist in the low μmolar range (Casper et al., 1999; Ciolino et al., 1998; Ciolino and Yeh, 1999). Another study showed that resveratrol might not only inhibit 4-OHE_2-induced MCF-10A transformation through its ability to function as an AhR antagonist, but also through its inhibiting effects on NF-κB signaling (Park et al., 2012). Similar to resveratrol, LicA inhibits oxidative estrogen metabolism in the low μM range, most likely through inhibiting AhR signaling and possibly through its anti-inflammatory effects. Further studies will determine if LicA can also reduce E_2-induced malignant transformation in MCF-10A cells similar to resveratrol. However, antagonization of AhR could potentially cause elongated half-life of compounds primarily metabolized by P450 1A2.

Induction of phase II enzymes by natural compounds is another mechanism that reduces genotoxic estrogen metabolites. Sulforaphane (10 μM), for example, is a potent chemopreventive compound that induces phase II enzymes, downregulated P450 1B1

protein levels by 50% and reduced estrogen DNA adducts by 60% in MCF-10A cells. Reduction of DNA adducts by sulforaphane was also observed in experiments with siKeap1, which suggests that inducers of the Keap1/Nrf2 pathway can significantly inhibit estrogen oxidative metabolism through up-regulating enzymes that detoxify genotoxic estrogen quinones (Yang et al., 2013). GI and LicA both activated the Keap1/Nrf2/ARE pathway in different cell lines, resulting in significant up-regulation of NQO1 in Hepa1c1c7 hepatoma cells and moderate induction in MCF-10A cells (Hajirahimkhan et al., 2015). These additional chemopreventive activities may increase LicA's and GI's overall chemopreventive potential.

In contrast to the inhibitory effects of GI, licorice extracts GG and GU stimulated estrogen metabolism (Figure 21A). Interestingly, in spite of LigC's anti-inflammatory activity, it enhanced oxidative estrogen metabolism (Figure 21C) and P450 1B1 expression (Figure 22C). P450 1B1 induction by LigC is in line with Cuendet et al.'s results, which previously reported that LigC increases P450 1A1 in mammary tissues (Cuendet et al., 2010). XRE-luciferase reporter activity was measured to determine if LigC increased 2- and 4-MeOE$_1$ through activation of AhR. LigC (1.5-fold induction) did not significantly activate XRE-luciferase reporter activity compared to a 70-fold induction with TCDD (Figure 24). In support of these data, Wong et al. showed that LigC did not activate XRE-luciferase reporter activity or affect the binding of AhR to XRE, as analyzed with EMSA in MCF-7 cells (Wong et al., 2014). LigC is possibly a very weak AhR agonist and/or acts via numerous other AhR-independent pathways that regulate P450 1A1/1B1 induction (Sissung et al., 2006). Future mechanistic studies will explore the specific biological targets of LigC. The slight induction of estrogen metabolism seen with LigF (Figure 21C) may be

the result of isomerization of LigF to LigC over the three day incubation (Simmler et al., 2013a), especially since LigF had no effect on P450 1B1 gene expression after 24 h (Figure 22C).

Interestingly, GG and GU increased both 4-MeOE$_1$ and P450 1B1 mRNA levels (Figure 21A, Figure 22B). The concentration of LigC even if all of the glycosides were hydrolyzed (i.e. LigC equivalents) in GG and GU are still much lower than those of pure LigC tested (µM) suggesting that additional constituents in the licorice extracts that were not evaluated in these studies may also modulate P450 1B1-mediated estrogen oxidative metabolism, P450 1B1/1A1 levels, and activities. For example, dibenzoylmethane, which is known to be present in GG, increases P450 1A1 and 1B1 in HepG2 cells and in rat liver accompanied by AhR activation (MacDonald et al., 2001; Mancia et al., 2014). Furthermore, glycyrrhetinic acid increases protein expression of P450 1A1 in mice (Hu et al., 1999). Other bioactive compounds like Glabridin and Glabrene have also been reported to have inhibitory activities towards P450 enzynmes and Tyrosinase (Kent et al., 2002; Nerya et al., 2003). These data suggest that LigC may not solely contribute to the stimulating effect on estrogen oxidative metabolism seen with GG and GU.

In summary, this study compared the three medicinally used *Glycyrrhiza* species using well authenticated plant material and clearly showed the chemical and pharmacological differences between the *Glycyrrhiza* species. Therefore, this study highlights the importance of standardization of licorice extracts to species-specific bioactive compounds that are used in botanical dietary supplements for women's health. The varied effects of the *Glycyrrhiza* species on estrogen metabolism might be due in part to differences in the biological targets of the Michael acceptor chalcones, LicA and LigC.

Both LicA and LigC have similar chemopreventive (Hajirahimkhan et al., 2015) and anti-inflammatory effects (this study); however, LicA is a strong AhR receptor antagonist and virtually shuts down genotoxic estrogen metabolism, whereas LigC stimulates oxidative estrogen metabolism through an unknown biological target. Because LicA and GI inhibit genotoxic estrogen metabolism *in vitro*, are anti-inflammatory, and activate detoxification enzymes (Hajirahimkhan et al., 2015) our *in vitro* results suggest that GI could be a promising chemopreventive licorice extract for women's health.

5. MODULATION OF ESTROGEN OXIDATIVE METABOLISM BY HOPS AND BIOACTIVE COMPOUNDS

(Reprinted in part with formatting changes from: Wang S, Dunlap TL, Howell CE, Mbachu OC, Rue EA, Chen SN, Pauli GF, Dietz BM, and Bolton JL. “Hop (*Humulus Lupulus L.*) extract and 6-prenylnaringenin induce P450 1A1 catalyzed estrogen 2-hydroxylation” *Chemical Research in Toxicology* (Epub 2016))

5.1 Rationale and Hypothesis

Hormone therapy (HT) has been the standard treatment option for postmenopausal symptom relief for decades (Gelety and Judd, 1992). However, HT has been linked with increased breast cancer risk in a number of clinical trials including the Women’s Health Initiative (WHI) (Beral, 2003; Holmberg and Anderson, 2004; Rossouw et al., 2002). As a natural and perceived safe alternative to HT for postmenopausal system relief, botanical dietary supplements have been increasingly popular (Bailey et al., 2011; Jiratchariyakul and Mahady, 2013). However, rigorous interdisciplinary studies on the efficacy, potential toxicity, and health benefits of these botanicals continue to be in high demand.

Prolonged exposure to estrogens including HT increases breast cancer risk (Cavalieri and Rogan, 2014; Russo and Russo, 2006; Yager, 2015). The two major mechanisms of carcinogenesis are estrogen signaling (hormonal pathway) and metabolism of estrogens to reactive quinones (chemical pathway, Figure 29). Recently, a number of cohort studies analyzing the risk correlation between estrogen levels, estrogen metabolites, and breast cancer risk in postmenopausal women were conducted (Dallal et al., 2014; Falk et al., 2013; Fuhrman et al., 2012). The results indicated that higher estrogen levels were associated with increased risk of postmenopausal breast cancer,

while enhanced estrogen 2-hydroxylation suggested a lower risk for breast cancer. Various *in vitro* studies have supported this finding that estrogen 2-hydroxylation represents a detoxification pathway whereas 4-hydroxylation is correlated with malignant transformation (Lakhani et al., 2003; Zahid et al., 2006; Ziegler et al., 2015).

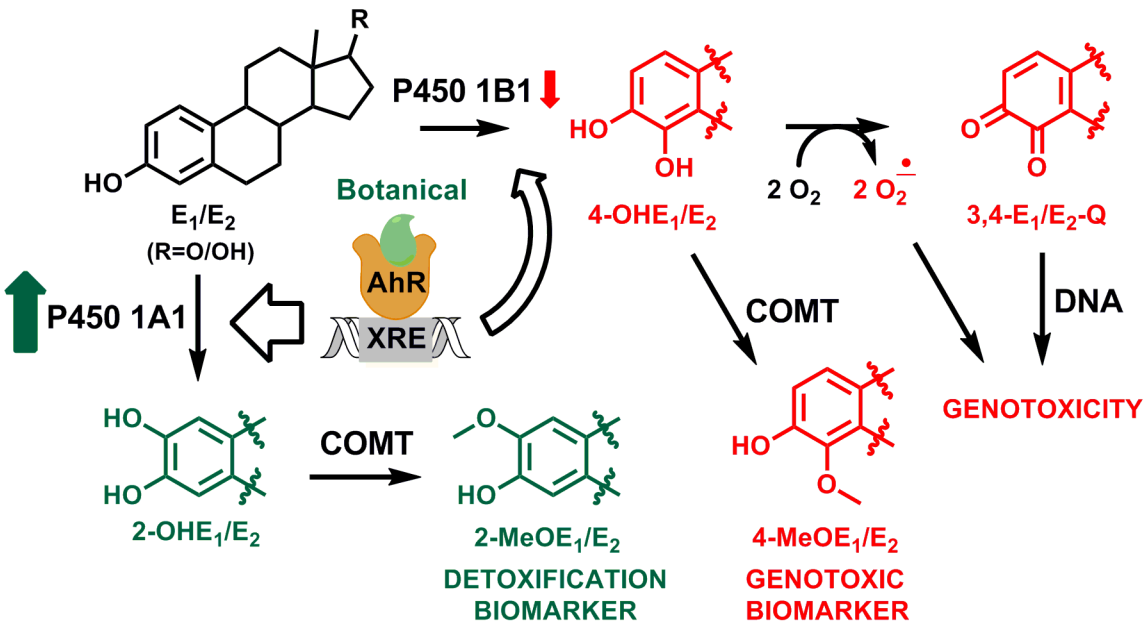


Figure 29. Hypothesis: effect of botanicals on estrogen chemical carcinogenesis. P450 1A1 catalyzes formation of 2-OHE$_1$/E$_2$ (detoxification biomarker), which are clinically shown to be correlated with reduced breast cancer risk. P450 1B1 catalyzes formation of 4-OHE$_1$/E$_2$ (genotoxic biomarker), which are oxidized to the genotoxic estrogen-3,4-quinone (3,4-E$_1$/E$_2$-Q) and forms ROS through redox cycling. The reactive quinone and ROS contribute to estrogen carcinogenesis. Chemopreventive botanicals are hypothesized to increase 2-hydroxylation metabolism and decrease 4-hydroxylation metabolism, as shown with green and red arrows.

P450 1A1/1B1 are the major extra-hepatic P450 1 enzymes that metabolize estrogens into 2- or 4-hydroxylated forms, respectively (Figure 29) (Lee et al., 2003). The expression is mainly controlled by the upstream aryl hydrocarbon receptor (AhR), which translocates into the nucleus upon activation and binds to xenobiotic response element (XRE), initiating targeted gene transcription (Nebert et al., 2004). In breast tissues, these P450s are directly correlated with the local estrogen metabolism (Cavalieri and Rogan, 2014; Russo and Russo, 2006; Yager, 2015). The 2- and 4-hydroxylated estrogen catechols can be further metabolized to the more stable 2- and 4-methoxy ether metabolites catalyzed by catechol-O-methyl transferase (COMT), which can be used as biomarkers for 2- and 4-hydroxylation pathways (Madhubhani et al., 2012; Xu and Veenstra, 2012).

Hops (strobili of *Humulus lupulus L.*, Cannabaceae) have been traditionally used as a sleep aid and, more recently, by women for postmenopausal symptom relief (Aghamiri et al., 2015; Keiler et al., 2013). Hops has been previously shown to have potent estrogenic activity with alkaline phosphatase induction, ERE activation, as well as ER down stream mRNA expression (Hajirahimkhan et al., 2013b). Many biological activities of hops have been connected to a series of bioactive prenylated flavanones and chalcones such as 6-prenylnarigenin (6-PN), 8-prenylnarigenin (8-PN), isoxanthohumol (IX), and xanthohumol (XH) (Figure 7) (Chadwick et al., 2004; Stevens and Page, 2004). The predominant prenylated chalcone, XH, has been shown to be an effective chemopreventive agent inducing the detoxification enzyme NAD(P)H:quinone oxidoreductase 1 (NQO1) in both *in vitro* and *in vivo* studies (Dietz et al., 2013; Gerhäuser et al., 2002; Stevens and Page, 2004). XH is metabolized to IX, desmethyl xanthohumol, 8-PN and 6-PN as shown in

Figure 7 (Legette et al., 2014; Nikolic et al., 2005). 8-PN has been reported to be one of the most potent estrogen receptor (ERα) phytoestrogens known to date, which is likely responsible for menopausal symptom relief (Chadwick et al., 2004; Milligan et al., 2000).

Previously, we showed that a hop extract was able to reduce the potentially genotoxic estrogen 4-hydroxylation pathway and decrease estradiol (E_2) induced colony formation in human non-tumorigenic MCF-10A cells (Madhubhani et al., 2012). The UIC/NIH Center for Botanical Dietary Supplements Research has further enriched a spent hop extract with respect to its estrogenic (8-PN) and chemopreventive (XH) compounds (Figure 7) (Krause et al., 2014; Ramos Alvarenga et al., 2014). The new extract contained much higher levels of the marker compounds than the previous extract. The purpose of this study was to test the effect of the first standardized hop extract, which has been used in human clinical trial, on estrogen metabolism (van Breemen et al., 2014). Since the relatively low response of non-tumorigenic breast epithelial MCF-10A cells was observed, we also included the well characterized breast MCF-7 cell line to confirm the bioactivities of the standardized hop extract as well as the effects of the four major prenylated marker compounds. The effects of this new hop extract and bioactive compounds on estrogen oxidative metabolism in the two breast cell lines was studied. Their effects on P450 1A1/1B1 enzymes, mRNA expression and EROD activity was also measured. Finally, XRE activation was analyzed in both liver HepG2 cells and MCF-7 cells to confirm the mechanism of action. The results suggest that hops can selectively enhance P450 1A1 catalyzed estrogen 2-hydroxylation and potentially reduce breast cancer risk.

5.2. Results

5.2.1. Hop extract and 6-PN preferentially induced estrogen 2-hydroxylation metabolism in MCF-10A and MCF-7 cells.

Previously we showed that a hop extract slightly decreased the estrogen 4-hydroxylation pathway and had no effect on the 2-hydroxylation pathway in MCF-10A cells in a 6 day experiment (Madhubhani et al., 2012). In the current study, two day metabolism studies with the new clinical hop extract described in the Material and Methods showed that this hop extract stimulated estrogen 2-hydroxyaltion (Figure 30A). One problem with MCF-10A cell line is its relatively low activities of P450 1A1/1B1 (Spink et al., 1998). In order to confirm the qualitative effect of hop extract and the bioactive marker compounds, additional experiments were done with the well characterized MCF-7 cells, which are known to be much more sensitive for P450 1A1/1B1 inductions (Spink et al., 1998). The data showed much higher overall induction of metabolism (10 - 20-fold), and 2-MeOE$_1$ formation was preferred similar to the MCF-10A data (Figure 30B). Although the preferential induction was observed in both cell lines, the differences in MCF-10A cells were more prominent than in MCF-7 cells with both hops and 6-PN treatment. 6-PN was the most potent compound tested inducing 2-MeOE$_1$ 50-fold compared to 40-fold for 4-MeOE$_1$ in MCF-7 cells and 3.5-fold for 2-MeOE$_1$ induction versus 2-fold for 4-MeOE$_1$ in MCF-10A cells (Figure 30C, 30D, 31). In contrast to the MCF-10A cell experiments, 8-PN showed moderate induction of estrogen metabolism in MCF-7 cells, which could be due to the higher AhR mediated P450 1A1/1B1 induction in MCF-7 cells. XH and IX did not have significant effects in either cell line. Overall, the results in MCF-7 cells are comparable with the MCF-10A data and suggested that hops and 6-PN preferentially induce estrogen 2-hydroxylation metabolism in breast cells.

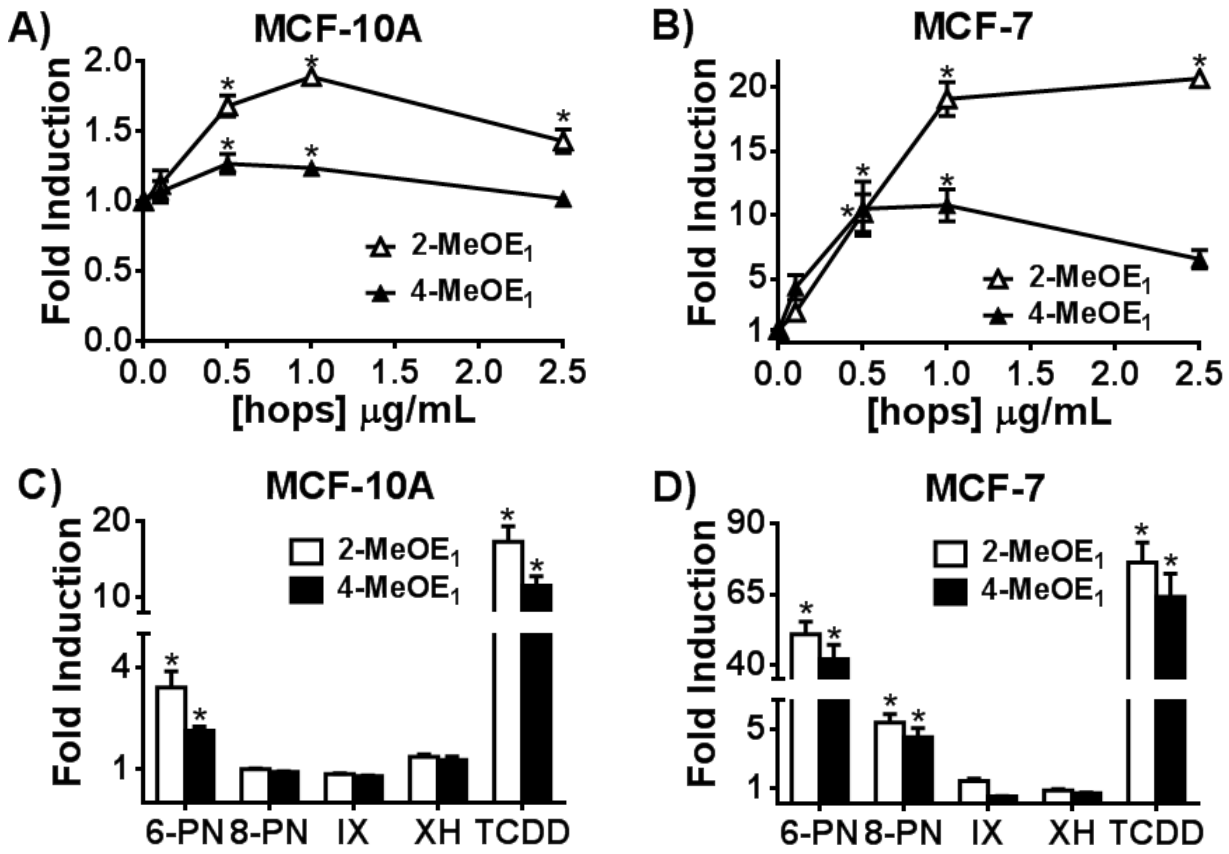


Figure 30. The hop extract and 6-PN preferentially induced 2-hydroxylation metabolism in breast MCF-10A and MCF-7 cells. A) MCF-10A cells and B) MCF-7 cells were treated with E_2 (1 μM) and the hop extract for 2 days and media were collected and analyzed for 2-$MeOE_1$ and 4-$MeOE_1$ metabolite level by LC-MS/MS. C) MCF-10A cells and D) MCF7 cells were treated with E_2 (1 μM) and 6-PN, 8-PN, IX, XH (1 μM), and TCDD (10 nM) for 2 days and media were analyzed for 2-$MeOE_1$ and 4-$MeOE_1$ metabolites. Results were normalized to fold induction against estradiol treated cells. Data were plotted as the means ± SEM of three independent experiments and analyzed by one-way ANOVA with Dunnett's multiple comparison post-test to compare treatment groups with control group, *$p < 0.05$.

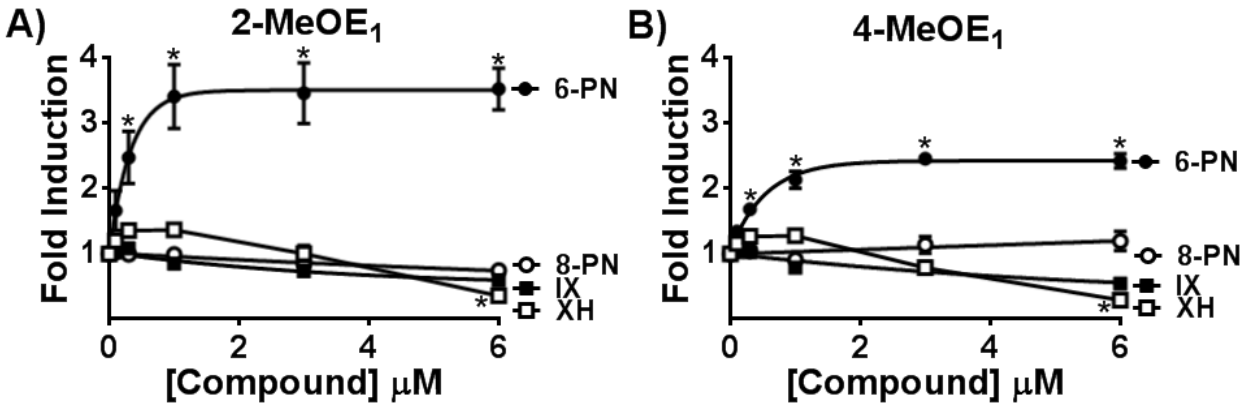


Figure 31. 6-PN dose-responsively and preferentially increased estrogen 2-hydroxylation in MCF-10A cells. MCF-10A cells were treated with the hop marker compounds in the presence of E_2 (1 μM) for 2 days and media were analyzed for 2-$MeOE_1$ and 4-$MeOE_1$ metabolites. Results were normalized to fold induction against estradiol treated cells. Data were plotted as the means ± SEM and analyzed by one-way ANOVA with Dunnett's multiple comparison post-test to compare treatment groups with control group, *$p < 0.05$.

5.2.2. Hop extract and 6-PN preferentially induced P450 1A1 mRNA expression in MCF-10A and MCF-7 cells.

P450 1A1/1B1 mRNA levels were analyzed 24 h after treatment with hops and the bioactive compounds. In MCF-10A cells, hop extract significantly induced P450 1A1 and 1B1 mRNA expression to 7 and 2-fold, respectively (Figure 32A). In MCF-7 cells, the induction levels were significantly higher with 90 and 35-fold induction of P450 1A1 and 1B1 mRNA expression (Figure 32B). In response to the hop compounds, the only compound that significantly increased P450 1A1 and 1B1 was 6-PN to 16 and 2-fold, respectively in MCF-10A cells (Figure 32C). In MCF-7 cells, qPCR analysis also showed that 6-PN preferentially increased P450 1A1 mRNA levels to around 290-fold compared to 25-fold induction of P450 1B1 (Figure 32D). 8-PN gave less significant induction: 90-fold

for P450 1A1 and 20-fold P450 1B1 mRNA expression, respectively. IX and XH did not show significant effects in either cell lines. Although these prenylated polyphenols share some common structural moieties, their bioactivities show remarkable differences. These data correlate with the results from estrogen oxidative metabolism, indicating that hops and 6-PN preferentially increased P450 1A1 mRNA levels in breast cells.

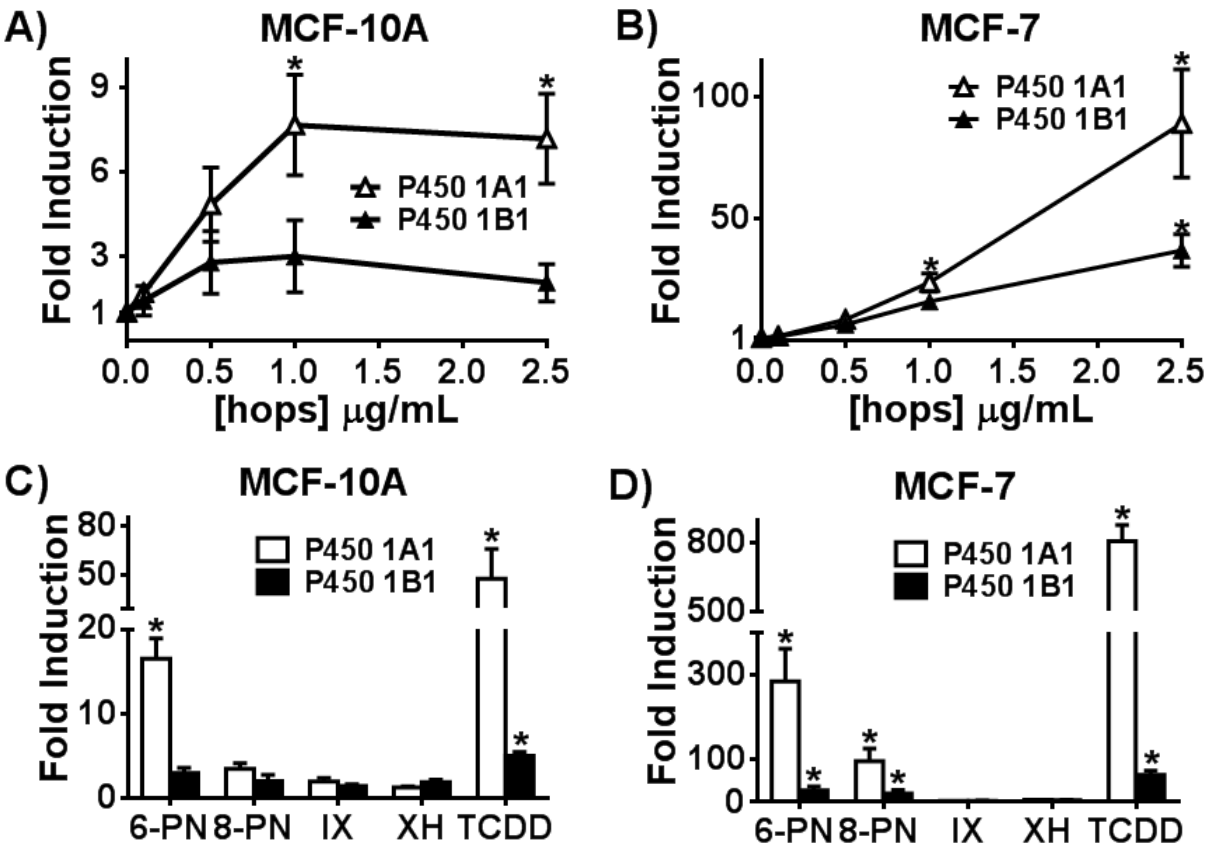


Figure 32. The hop extract and 6-PN preferentially induced P450 1A1 mRNA expression in MCF-10A and MCF-7 cells. A) MCF-10A cells and B) MCF-7 cells were treated with hop extract and P450 1A1 and 1B1 mRNA expression were analyzed after 24 h via qPCR. C) MCF-10A and D) MCF-7 cells were treated with 6-PN, 8-PN, IX, XH (1 μM), and TCDD (10 nM) for 24 h and P450 1A1/1B1 mRNA expression were analyzed via qPCR. Results were plotted as the means ± SEM of three independent experiments and analyzed by one-way ANOVA with Dunnett's multiple comparison post-test to compare treatment groups to DMSO control, *$p < 0.05$.

5.2.3. Hop extract and 6-PN induced P450 1A1/1B1 activity in MCF-10A and MCF-7 cells.

P450 1A1/1B1 activity was measured using the EROD assay in both cell lines after 2 days of treatment with hop extract and bioactive compounds. In MCF-10A cells, in the presence of the hop extract, a significant dose-responsive induction was observed to a maximum of 0.04 pmole/min/well resorufin formed (Figure 33A). In MCF-7 cells, the hop extract gave significantly higher EROD activity compared to MCF-10A cells to a maximum of 0.25 pmole/min/well resorufin (Figure 33B). With the hop compounds in MCF-10A cells, only 6-PN increased P450 1A1/1B1 activity dose-dependently to 0.05 pmole/min/well resorufin (Figure 33C). XH moderately induced the P450 activity even though estrogen metabolism and P450 1A1/1B1 gene expression were not affected; 8-PN and IX did not show significant effects. In MCF-7 cells, significant induction of P450 1A1/1B1 activity were observed with 6-PN and 8-PN (3 μM) to 0.8 and 0.2 pmole/min/well resorufin, while IX and XH did not have significant effects (Figure 33D). Overall, the results from the EROD activity assay were consistent with the results from estrogen metabolism and P450 1A1/1B1 mRNA analysis. Qualitatively, the data from these two cell lines was also comparable and indicated that hops and 6-PN strongly induced P450 1A1/1B1 activity in breast cells.

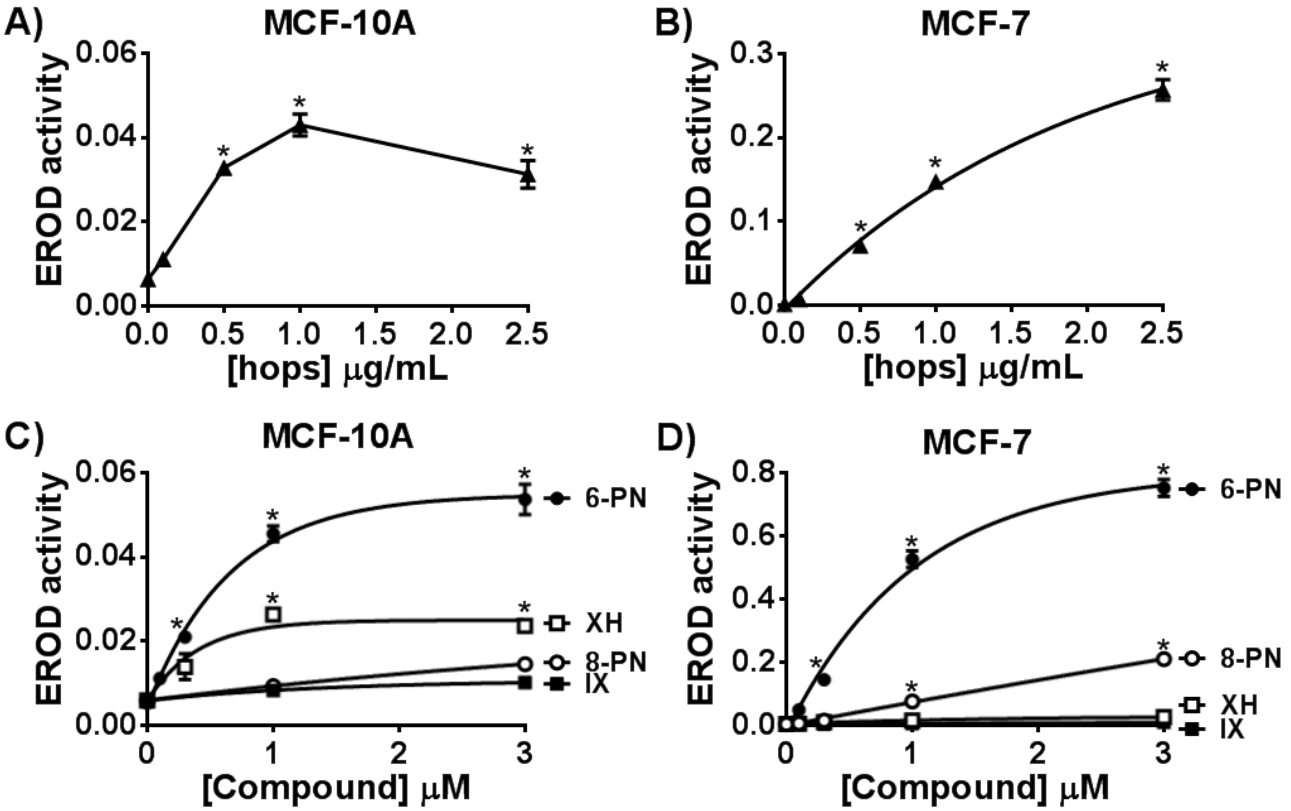


Figure 33. The hop extract and 6-PN increased P450 1A1/1B1 activity in MCF-10A and MCF-7 cells. P450 1A1/1B1 activity was analyzed in MCF-10A cells after 2-day treatment of A) hop extract and C) different doses of 6-PN, 8-PN, IX, and XH with EROD assay. P450 1A1/1B1 activity was analyzed in MCF-7 cells after 2-day treatment of B) hop extract and D) different doses of 6-PN, 8-PN, IX, and XH with EROD assay. Results were represented as pmole/min/well resorufin formed and plotted as the means ± SEM of three independent experiments and analyzed by one-way ANOVA with Dunnett's multiple comparison post-test to compare treatment groups to DMSO control, *$p < 0.05$.

5.2.4. 6-PN increased XRE activation and acted as AhR agonist.

Human hepatoma HepG2 cells and MCF-7 cells transfected with a XRE luciferase construct were used to measure the effect of bioactive compounds on AhR activation. The cells were incubated with the compounds for 24 h after transient transfection of the

luciferase construct. Of the four polyphenols, 6-PN and 8-PN significantly and dose-dependently increased XRE-luciferase activity to around 6.5 and 10-fold of control at 10 μM, while XH and IX did not have significant effects (Figure 34A). In the presence of the AhR agonist TCDD (10 nM), a dose-responsive decrease in XRE luciferase activity was observed with 6-PN and 8-PN co-treatment (Figure 34B), which further suggested the interactions with AhR. In MCF-7 cells, the XRE response was lower than that in HepG2 cells (Figure 34C). 6-PN (5 μM) significantly induced the activity while 8-PN (5 μM) had no effect, which correlated with the P450 1A1/1B1 activity and gene expression analysis. The results indicated some cell selectivity of 8-PN and also suggested 6-PN to be activating AhR in both breast and liver cell lines. Lastly, upon co-treatment of AhR antagonist CH223191 and 6-PN (1 μM) in MCF-7 cells, the EROD activity was inhibited dose responsively (Figure 34D), which further confirmed 6-PN to be an AhR agonist. In summary, these results suggested that 6-PN acted as an AhR agonist in both breast and liver cells.

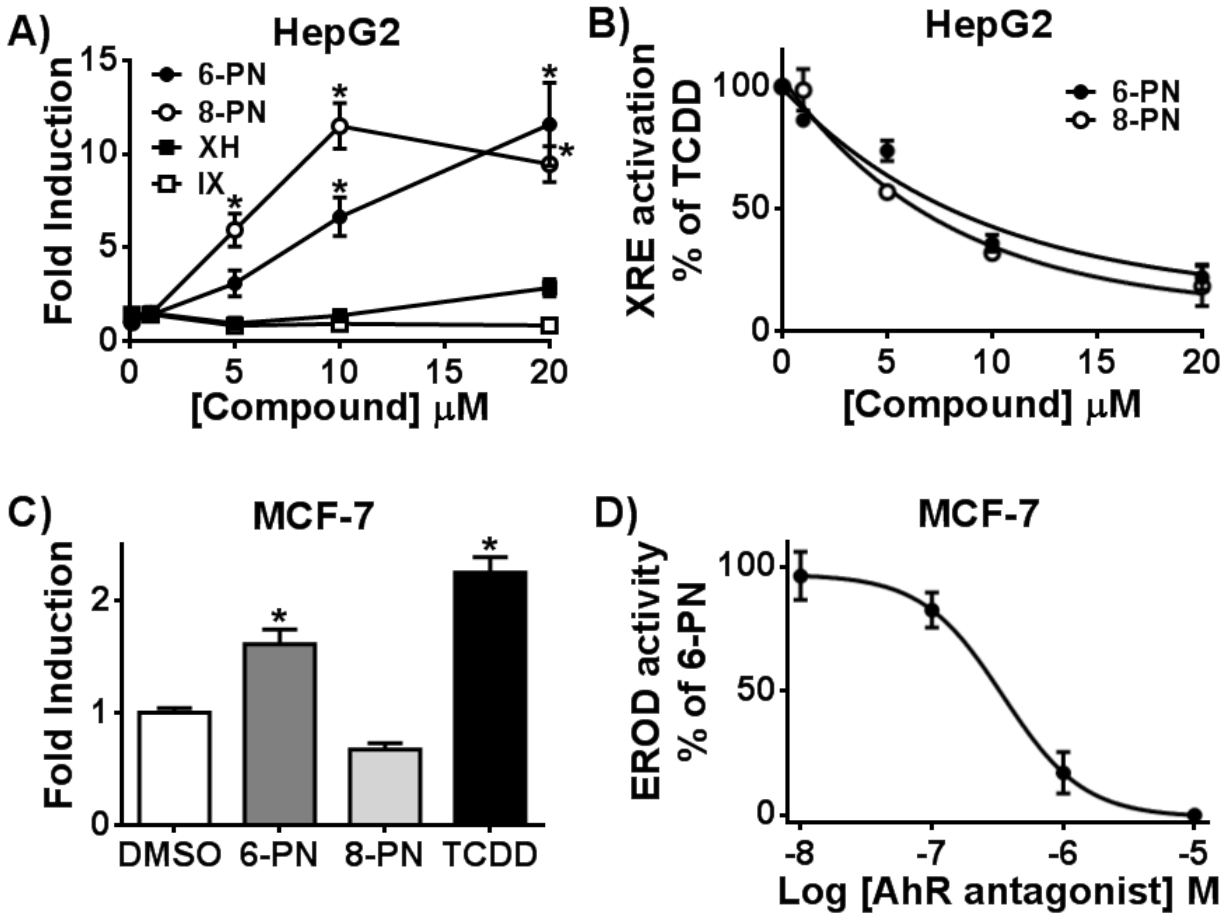


Figure 34. 6-PN induced XRE-luciferase activity and acted as a partial AhR agonist. HepG2 cells were incubated with A) hop compounds alone and B) 6-PN and 8-PN in the presence of TCDD (10 nM) for 24 h before analysis of XRE-luciferase reporter activity. C) MCF-7 cells were incubated with 6-PN, 8-PN (5 μM), and TCDD (10 nM) for 24 h before analysis of XRE-luciferase reporter activity. D) P450 1A1/1B1 activity was measured via EROD assay in MCF-7 cells after co-treatment of 6-PN (1 μM) with AhR antagonist CH223191 (0.01, 0.1, 1, and 10 μM) for 2 days. Results were plotted as the means ± SEM of three independent experiments and analyzed by one-way ANOVA with Dunnett's multiple comparison post-test to compare treatment groups to control group, *$p < 0.05$.

5.2.5. The hop compounds inhibit recombinant P450 1A1/1B1 activity, but had only moderate P450 1A1/1B1 inhibition in cells.

Various flavonoids have been previously reported to inhibit P450 1A1/1B1 activities (Chaudhary and Willett, 2006; Takemura et al., 2010; Zhai et al., 1998). To study the inhibitory effects of hop compounds on P450 1A1/1B1, EROD assay with recombinant enzymes were conducted. All four compounds acted as P450 1A1/1B1 inhibitors with IC_{50} values in the low μM range without selectivity for either P450 1A1 or 1B1 (Table III). However, the inhibitory activity of these compounds in a cell culture environment was considerably less than with recombinant enzymes (Figure 35 & 36). These data indicated the metabolism results were mainly the result of induction of P450 1A1/1B1 enzyme levels and little direct inhibition of P450s should be observed at clinical concentrations of hop supplements.

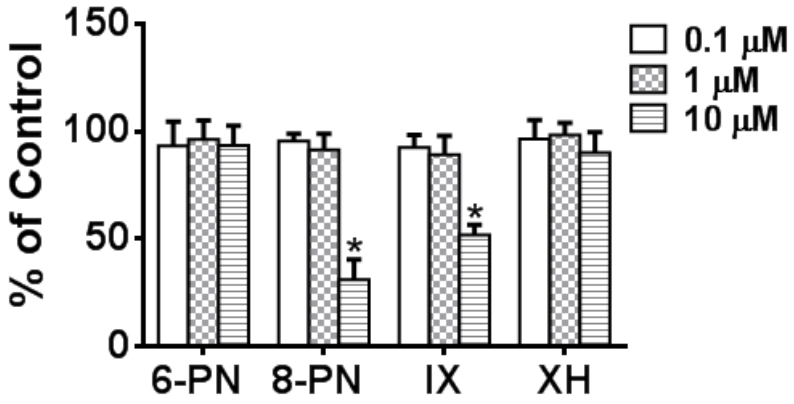


Figure 35. Hop compounds moderately inhibited P450 1 activity in cells. MCF-7 cells were pretreated with TCDD (10 nM) for 48 h and pre-incubated with 6-PN/8-PN/IX/XH (0.1 μM, 1 μM, and 10 μM) for 5 min at 37°C before ethoxyresorufin and NADPH was added for 20 min. EROD activity was measured and results were analyzed by one-way ANOVA with Dunnett's multiple comparison post-test, * $p < 0.05$.

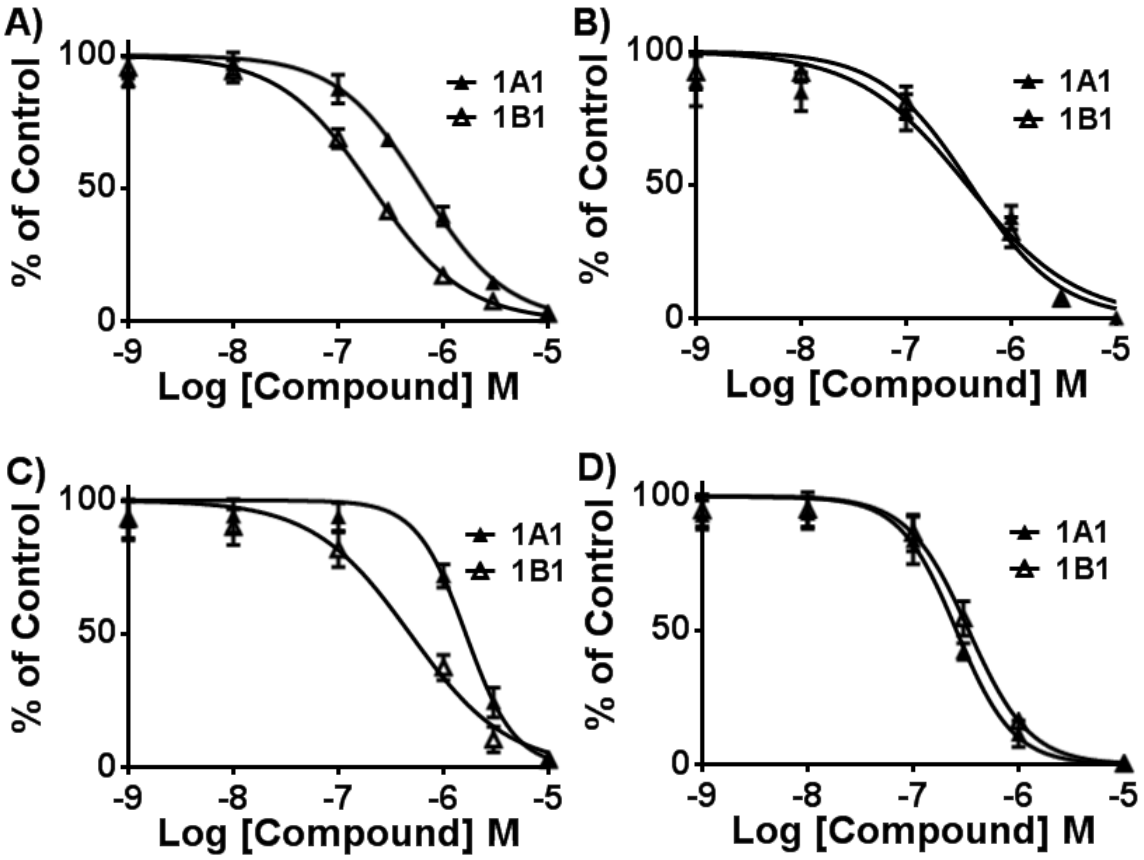


Figure 36. Hop compounds inhibited human recombinant P450 1A1 and 1B1 activity. Human recombinant P450 1A1 and P450 1B1 protein with reductase were incubated with 7-ethoxyresorufin, NADPH and different doses of A) XH, B) IX, C) 8-PN and D) 6-PN at 37°C for 20 min. EROD activity was measured and data were plotted as percentage of activity against vehicle control.

Table III. Inhibition of recombinant P450 1A1/1B1 enzymes with hop compounds.[a]

	IC_{50} (μM)			
	6-PN	**8-PN**	**IX**	**XH**
P450 1A1	0.63 ± 0.08	0.38 ± 0.12	1.6 ± 0.17	0.28 ± 0.03
P450 1B1	0.21 ± 0.02	0.41 ± 0.08	0.48 ± 0.11	0.34 ± 0.04

[a]The IC_{50} values are expressed as means ± SD from three independent dose-responsive curves using human recombinant P450 1A1 and P450 1B1 enzymes.

5.2.6. 6-PN down-regulated ER expression in MCF-7 cells.

Alongside of estrogen chemical carcinogenesis, ER signaling determines the activity of estrogen hormonal carcinogenesis pathway. By inhibiting ER signaling, the estrogen hormonal carcinogenesis pathway activity could be reduced for breast cancer prevention. Other than the traditional SERMs that acts through ER binding, ER down-regulation has also been shown to significantly decrease ER signaling (Bross et al., 2003).

ER down-regulation mediated through the AhR signaling activation has also been previously reported (Matthews and Gustafsson, 2006; Wormke et al., 2000) (Figure 37). The down-regulation effect was proposed to be caused by untargeted ubiquitination triggered by AhR activation. When co-treated with a proteasome inhibitor, ER down-regulation was attenuated (Tiong et al., 2012b; Wormke et al., 2000). Additionally, *in vivo* studies also showed inhibitory effect of AhR agonists on ER signaling. Dr. Guenter Vollmer group showed that 3-MC, which is an AhR agonist, showed anti-estrogenic activity *in* vivo through decreasing ER protein and signaling. 3-MC inhibited E_2-induced terminal end buds development and ER target protein/gene expression in the mammary tissues (Helle et al., 2016).

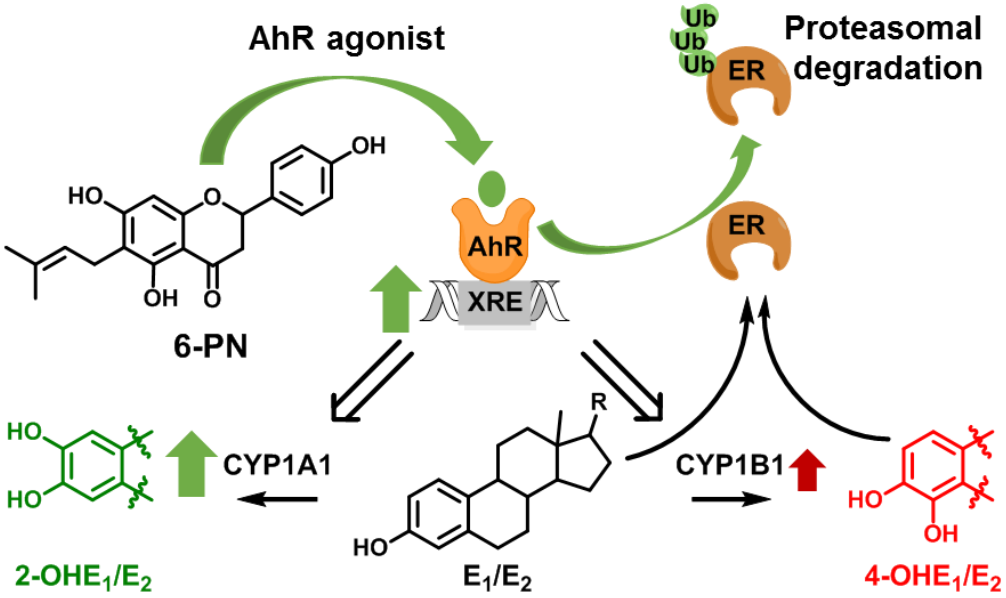


Figure 37. Scheme of ER down-regulation by AhR activation. AhR activation triggers untargeted ubiquitination of ER. The ubiquitinated ER then goes through proteasomal degradation. ER ubiquitination can also be induced by receptor activation after binding with ER ligands, such as estradiol and 4-hydroxyestradiol.

Since 6-PN is a potent AhR agonist discovered in hops, its action on the ER was also evaluated. After normalization to control treatment as 100% and fulvestrant (1 μM) treatment as 0%, in MCF-7 cells, dioxin like pure AhR agonist TCDD reduced the ER protein level to around 60%, and treatment with E_2 reduces the ER protein level to around 50% (Figure 38A). Co-treatment of TCDD and E_2 showed additive effects on ER down-regulation. AhR activity and estrogenicity contributed about equal to the down-regulation to around 20%. Previous reports with PAH AhR agonist 3-MC showed that it down-regulated ER expression and also showed μM estrogenic activity (Abdelrahim et al., 2006). In this experiment, 3-MC decreased ER expression to around 20% at 10 μM, which was consistent with previous published results (Tiong et al., 2012b); 6-PN had similar effects to

3-MC at 5 µM. Additionally, 6-PN dose-responsively down-regulated ER with similar potency and efficacy to 3-MC. Since 6-PN was found to be an AhR agonist from this study and previously shown to also have µM estrogenic activity (Milligan et al., 2000), , the down-regulation by 6-PN is probably mediated through both AhR activation and partial estrogenic activity (Figure 38B). Thus, 6-PN was dose-responsively reducing the ER level mainly through AhR activation at concentrations less than 1 µM; the down-regulation at concentrations higher than 1 µM was due to the effect of AhR agonist activity and partial estrogenicity.

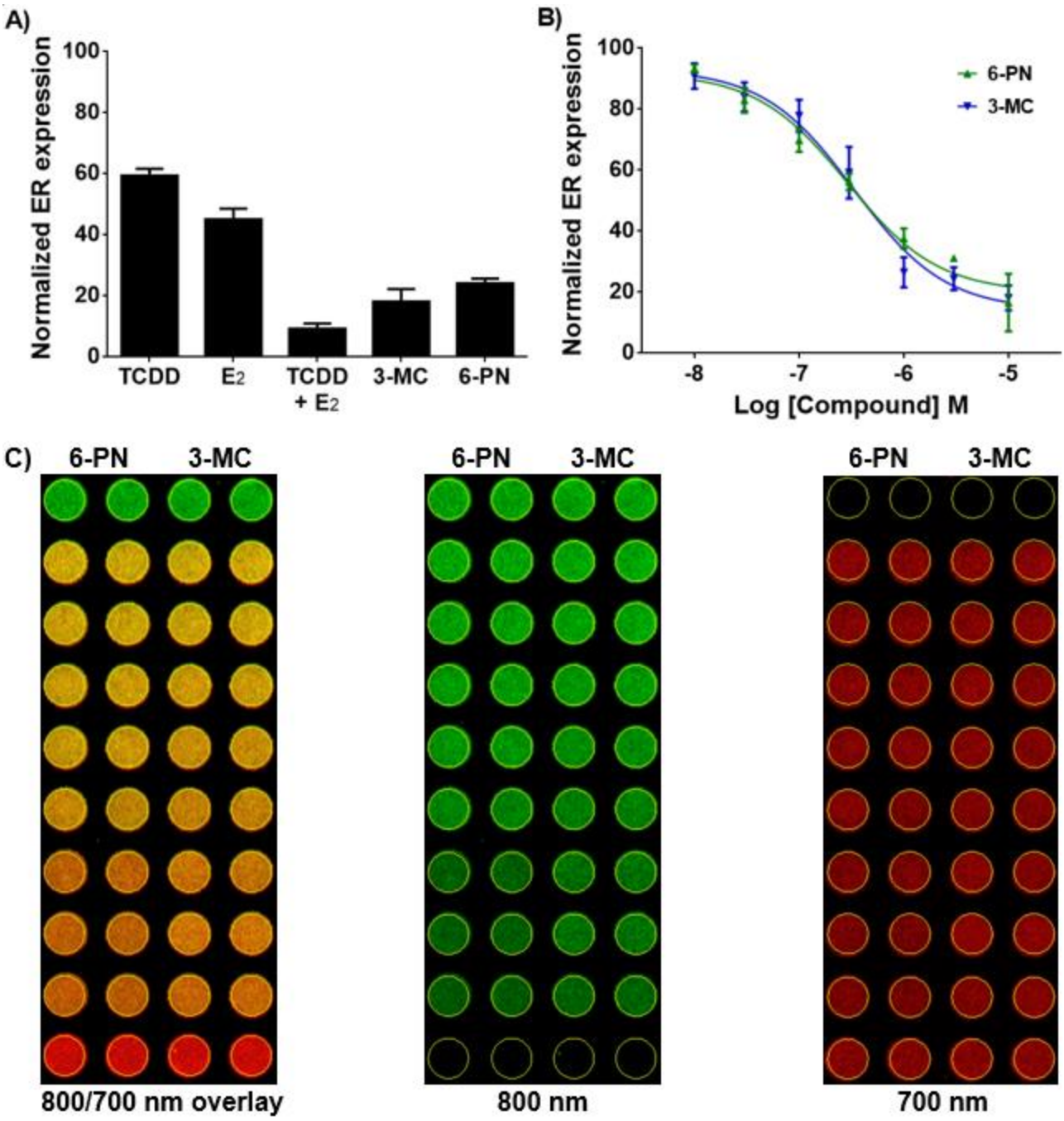


Figure 38. 6-PN down-regulated ER expression in MCF-7 cells. MCF-7 cells were estrogen starved for 24 h before treatment with A) TCDD (10 nM), E_2 (1 µM), TCDD (10 nM) + E_2 (1 µM), 3-MC (10 µM), or 6-PN (5 µM) B) different concentrations of 6-PN and 3-MC for 48 h. ER expression was measure using in-cell western and normalized to control treatment as 100% and fulvestrant (1 µM) as 0%. C) Representative dose response result of 6-PN and 3-MC on ER expression analyzed by LI-COR in cell western at 800 nm for ER and 700 nm for cell number normalization.

Previously, Tiong et al showed that the prenylated flavone, icaritin, reduced ER expression in MCF-7 cells through AhR activation (Tiong et al., 2012b). Icaritin at 1 µM was shown to induce P450 1A1 mRNA expression to around 30% of the induction by the control compound 3-MC. While, comparatively in our study, 6-PN induced P450 1A1 mRNA expression to around 40% of TCDD induction at 1 µM (Figure 32). Similarly, icaritin reduced ER expression to around 50% of control at 1 µM while 6-PN decreased ER to around 40% of control (Figure 38). Additionally, from the previous *in vivo* results, although being a carcinogen itself, AhR agonist 3-MC helped relieve the breast tumor formation through decreasing ER protein and ER signaling (Helle et al., 2016). Thus, the non-toxic AhR agonist 6-PN from hops could be used as a better alternative to reduce ER signaling for the prevention of estrogen hormonal carcinogenesis. Together with the activity in estrogen metabolism modulation, 6-PN and hops could present better activity in estrogen carcinogenesis prevention with less side effects.

In conclusion, 6-PN could help prevent estrogen carcinogenesis through preferential up-regulation of estrogen 2-hydroxylation metabolism and down regulation of ER. The dual actions on both estrogen chemical carcinogenesis pathway and hormonal carcinogenesis pathway would limit genotoxic metabolite formation and ER signaling at the same time. 6-PN and hops can be further tested in *in vivo* systems for both short term and long term effects on estrogen carcinogenesis.

5.3. Discussion

Estrogen exposure has long been linked with postmenopausal breast cancer risk, especially since the WHI report in 2002 (Rossouw et al., 2002; Russo and Russo, 2006;

Yager, 2015). Estrogen carcinogenesis includes the hormonal mechanism involving classical estrogen receptor binding as well as estrogen signaling which promotes cell growth and the chemical mechanism where estrogens are converted to reactive quinones which modify DNA leading to genotoxicity (Figure 29) (Cavalieri and Rogan, 2014; Russo and Russo, 2006; Yager, 2015). The estrogen 4-hydroxylation pathway is considered the genotoxic pathway by forming the electrophilic/redox active estrogen-3,4-quinone and reactive oxidative species (ROS) which causes DNA damage (Cavalieri et al., 2006; Park et al., 2009). Contrary to estrogen 4-hydroxylation, estrogen 2-hydroxylation is a non-genotoxic pathway and the metabolite 2-methoxyestradiol has been shown to have anti-proliferative/anti-cancer activity (Lakhani et al., 2003). Several recent clinical trials analyzing serum estrogen metabolite levels and the risk of postmenopausal breast cancer further support the beneficial 2-hydroxylation pathway in potentially decreasing breast cancer risk; however, the data is inconclusive on the relationship between the estrogen 4-hydroxylation pathway and breast cancer risk (Dallal et al., 2014; Falk et al., 2013; Fuhrman et al., 2012; Ziegler et al., 2015).

P450 1A1 and 1B1 are the major enzymes in breast tissues that are responsible for the local estrogen 2- and 4-hydroxylation metabolism (Huang et al., 1996; Williams and Phillips, 2000). These two enzymes share about 40% homology and are generally co-upregulated upon AhR activation (Tijet et al., 2006). AhR is also responsible for the expression of several other Phase I and Phase II enzymes, which are associated with detoxification of environmental carcinogens as well as potential activation of pro-carcinogens (Androutsopoulos et al., 2009). P450 1B1 has been shown to be over-expressed in various cancer cells, and environmental carcinogens induced P450 1B1 in

cancer cells/tumors was shown to be much higher than in normal cells/tissues (Beischlag et al., 2008; Gajjar et al., 2012; Yang et al., 2008b).

MCF-10A cells have been frequently used as a model to study estrogen chemical carcinogenesis due to the absence of estrogen receptor and non-tumorigenic phenotype (Chen et al., 2004; Dunlap et al., 2015; Russo et al., 2003; Santen et al., 2015; Yager, 2015). However, the AhR mediated P450 1A1/1B1 induction in MCF-10A cells is relatively low as reported previously and confirmed in the present study (Spink et al., 1998). Data also suggests that the Phase I and Phase II enzyme expression is variable in MCF-10A cells depending on confluence level in cell culture (Fu et al., 2011). In addition, spontaneous expression of ER might occur in MCF-10A cells after a certain number of passages (Lane et al., 1999). These potential problems and the variability observed in the current experiments prompted additional studies in the more robust MCF-7 cells.

MCF-7 cells have been used in estrogen metabolism studies by different groups (Huang et al., 2011; Santen et al., 2015; van Duursen et al., 2003). Spink et al. showed that induction of P450 1A1/1B1 enzymes vary among different tumorigenic and non-tumorigenic cell lines. They determined that MCF-7 cells had higher levels of TCDD-induced P450 1A1/1B1 mRNA and almost 8-fold higher metabolic rates for TCDD-induced 4-MeOE_2 formation compared to MCF-10A cells (Spink et al., 1998). Similarly, our results showed more than 15-fold increase in P450 1A1/1B1 gene induction with TCDD treatment in MCF-7 over MCF-10A cells (Figure 32C & 32D) and about 10-fold higher metabolite formation with hop extract treatment in MCF-7 compared to MCF-10A cells (Figure 30A & 30B). Since MCF-7 cells mirrored MCF-10A cells in the response to botanical treatment with higher induction levels, they serve as a better model to screen botanicals and

compounds for modulation of estrogen metabolism. In addition, the upstream P450 1A1/1B1 mRNA induction trend among compounds (6-PN>>8-PN and no effect with IX and XH) corresponds with the metabolism and activity results in MCF-7 cells. However, due to the presence of estrogen receptor there is potential crosstalk between AhR and ERα signaling pathways and AhR agonists have been reported to increase proteasomal degradation of ER (Tiong et al., 2012a; Wormke et al., 2003). The interesting relationship between ERα, AhR and botanical modulation of estrogen metabolism will be the subject of future studies.

Botanicals have been previously shown to affect AhR activation, and the major compounds responsible were polyphenols (Moon et al., 2006; Zhang et al., 2003). For example, resveratrol has been shown to induce XRE activation to about 6-fold at 10 µM in MCF-10A cells (Chen et al., 2004). In this study, HepG2 cells were used to compare the AhR activation by hop compounds, because the XRE-luciferase reporter activity in HepG2 cells were much higher compared to MCF-7 cells (Figure 39). HepG2 cells with ER transfected could also be used to better mimic the cellular protein expressions of MCF-7 cells (ref).

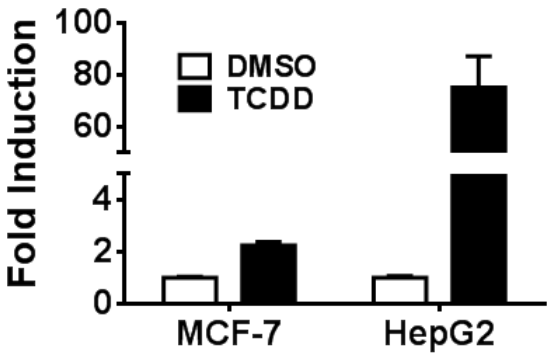


Figure 39. HepG2 cells had significantly higher response than MCF-7 cells to TCDD induced XRE-luciferase activity. MCF-7 cells and HepG2 cells were treated with TCDD (10 nM) after transfection for 24 h. Cells were then lysed and measured for luciferase as previously described.

Comparatively, in this study XRE luciferase activity was increased to 6.5-fold by 6-PN (10 µM) in HepG2 cells (Figure 34A). 8-PN displayed some cell selectivity with about 10-fold XRE activation at 10 µM in HepG2 cells, but showed no effect in MCF-7 cells. In addition, hop compounds also showed differential effects on P450 1A1/1B1 regulation. Preferential induction of P450 1A1 over P450 1B1 has been documented in the literature. Quercetin (10 µM) and berberine (>5 µM) preferentially induced P450 1A1 over 1B1 in MCF-10F and MCF-7 cells, respectively (Mense et al., 2008; Wen et al., 2014). On the contrary, benzo(*a*)pyrene (1 µM) preferentially increased P450 1B1 in human oral epithelial cells (Wen and Walle, 2005). However, the mechanisms of P450 1A1 and 1B1 preferential induction as well as *in vivo* effects need to be further studied. Significant P450 1A1/1B1 activity and mRNA expression increases were observed with 6-PN treatment. Induction of XRE activity and inhibition of TCDD induced XRE activity further supported 6-PN to be an AhR agonist (Figure 34). Induction of P450 1 enzymes by natural flavonoids

and flavonoid rich botanicals have been reported previously (Moon et al., 2006). Naringenin, a flavanone with the same scaffold as the hop flavanones, does not induce P450 1A1/1B1 (Allen et al., 2001; Zhang et al., 2003), while the effect of flavanones with a prenylated side chain has not been studied (Gross-Steinmeyer et al., 2004). Results from this study indicated that prenylated naringenin derivatives exhibit a unique activity compared to the parent naringenin. In addition, the significant P450 1A1/1B1 induction by 6-PN compared to 8-PN may suggest that the positioning of the prenyl group is important for AhR activation.

The effect of hop compounds on the activation of AhR in the presence of TCDD were measured as P450 1A1/1B1 enzymes can also be regulated independently of AhR (Hu et al., 2007; Šmerdová et al., 2014). The results indicated that 6-PN and 8-PN had comparable effects in inhibiting TCDD-induced XRE luciferase activity at μM range (Figure 34B). Resveratrol, as well as the scaffold parent compound naringenin, also demonstrated significant inhibitory effects on TCDD induced EROD activity above 10 μM in MCF-10A cells (Allen et al., 2001; Chen et al., 2004). Similar studies looking at estrogen metabolism using a MCF-10F cell model observed decreased formation of 4-MeOE_1/E_2 and DNA adducts after co-treatment with resveratrol (25 μM) and TCDD (10 nM) (Lu et al., 2008). In contrast, we previously showed that licochalcone A (10 μM), a gamma-prenylated chalcone from licorice (*Glycyrrhiza inflata*), was an AhR antagonist and shut down estrogen oxidative metabolism in MCF-10A cells (Dunlap et al., 2015).

Various botanical flavonoids have been identified as inhibitors of P450 enzymes (Chaudhary and Willett, 2006; Takemura et al., 2010; Zhai et al., 1998). It has also been shown that flavonoids generally exhibit more potent P450 1B1 inhibition over 1A1

(Shimada et al., 2010). Several ubiquitous, unsubstituted flavones and flavonols, such as quercetin, kaempferol and apigenin, have been reported to be P450 1B1 inhibitors with IC_{50} values below 50 nM, while inhibiting P450 1A1 activity less potently (Shimada et al., 2010; Takemura et al., 2010; Zhai et al., 1998). However, flavanones, like narigenin, are generally weak P450 1 inhibitors with IC_{50} values in the μM range, which might indicate the importance of the 2-3 double bond in P450 1 enzyme inhibition (Leung et al., 2007; Takemura et al., 2010). Several hydroxychalcones have been tested for the inhibition of P450 1A1 and 1B1 and showed IC_{50} values in the low μM range (Wang et al., 2005). Our results as well as previous studies from Henderson et al. showed that the prenylflavanones from hops are more potent inhibitors compared to the parent flavanone, naringenin (Henderson et al., 2000). The hop compounds had similar inhibitory activities towards P450 1B1, with IC_{50} values around 0.5 μM; 6-PN showed around 3-fold lower inhibitory activity to P450 1A1 (IC_{50} 0.6 μM) than 1B1 (IC_{50} 0.2 μM) (Table III). When compared to resveratrol, which inhibited P450 1A1 and 1B1 with IC_{50} values around 2 and 25 μM respectively, these hop compounds are more potent P450 1 inhibitors (Chen et al., 2004). The hop compounds have also been shown to inhibit P450 2C8, 2C9 and 19 with IC_{50} values in the low μM range (Monteiro et al., 2006; Yuan et al., 2014). However, in contrast to the experiments with purified P450s, the hop compounds showed little to no inhibition in cells (Figure 35), likely due to extensive metabolism (Legette et al., 2014; Nikolic et al., 2005). These data suggests that the effect on estrogen oxidative metabolism modulation was mainly contributed by the AhR agonist activity of 6-PN. The results from this study suggests that hop extracts should not only be standardized to 8-PN for estrogenic activity and to XH for chemopreventive activity, but also to 6-PN for its potential effect on estrogen

metabolism. However, further studies are needed to test and confirm the activities *in vivo* (Possemiers et al., 2006; van Breemen et al., 2014).

In conclusion, results from this study provided novel *in vitro* evidence that hops and its compound 6-PN preferentially induced the non-toxic estrogen 2-hydroxylation pathway in two different breast cell lines, which indicated a potentially protective role of hops to help reduce the risk of breast cancer through estrogen metabolism modulation. As hop dietary supplements are taken widely by women for postmenopausal symptom relief, it is important to expand our knowledge about the bioactivity and safety of 6-PN and related hop compounds. The clinical trial data has indicated long half-lives of these hop compounds (van Breemen et al., 2014). Since the pharmacokinetic properties of the hop compounds would significantly influence their effect in vivo, the preferential 2-hydroxylation induction as well as modulation of P450 1A1/1B1 enzymes would need to be tested with *in vivo* models that will be studied in the future. The present data also confirm the importance of performing botanical standardization to several bioactive phyto-constituents simultaneously. For hop extracts, suitable target markers are 8-PN (estrogenic), XH (chemopreventive), and 6-PN as modulator of estrogen metabolism. The levels of these compounds need to be measured for the health and safety of women taking hop extract, which will provide a basis for standardization of safe and effective hop botanical supplements.

6. CONCLUSIONS AND FUTURE DIRECTIONS

Botanical modulation of estrogen oxidative metabolism, which is involved in estrogen chemical carcinogenesis, was evaluated in this study with botanical dietary supplements used for women's health. It was hypothesized that botanicals might decrease breast cancer risk through inhibition of the genotoxic pathway and/or through induction of the detoxification pathway of estrogen oxidative metabolism. *In vitro* MCF-10A and MCF-7 breast cell lines were used in this study. The formation of 2- and 4- methoxyestrone was measured by LC-MS/MS as markers for the estrogen 2- and 4-hydroxylation pathway after treatment of the breast cells with estradiol and botanicals. The activity and expression of P450 1A1 and 1B1 enzymes, which catalyze estrogen 2- and 4-hydroxylation, respectively, in breast cells, were determined to test if the influence on the metabolism was mediated by these enzymes. The upstream aryl hydrocarbon receptor which controls the expression of P450 1A1 and P450 1B1, was also studied to understand the underlying mechanisms. Further, identified active bioactive compounds and botanicals were tested in ACI rat models to determine their actions in the *in vivo* system.

Two botanical species were found to have beneficial activity in modulating estrogen oxidative metabolism. Licorice *Glycyrrhiza inflata* (GI) showed activity in inhibiting estrogen 4-hydroxylation carcinogenic pathway through down-regulation of P450 1B1 enzyme while the other two commonly used licorice species *Glycyrrhiza glabra* (GG) and *Glycyrrhiza uralensis* (GU) slightly induced the estrogen oxidative metabolism. The GI specific chalcone licochalcone A (LicA) was later determined to be responsible for the activity of GI in down-regulating estrogen 4-hydroxylation metabolism. LicA dose responsively down-regulated estrogen oxidative metabolism through down-regulation of P450 1B1 enzyme

expression, which was achieved through AhR inhibition. In the *in vivo* ACI rat model, LicA also showed expected effects and down-regulated estrogen oxidative metabolism.

Hops extract showed preferential induction on the estrogen 2-hydroxylation pathway in both non-tumorigenic MCF-10A cells while not affecting the 4-hydroxylation pathway. In tumorigenic MCF-7 breast cells, hops extract still has activity in preferential up-regulation of 2-hydroxylation pathway in the expense of moderate induction of 4-hydroxylation pathway. The hop compound 6-prenylnaringenin (6-PN) showed corresponding activity to hops that it preferentially upregulated 2-hydroxylation metabolism through preferential up-regulation of P450 1A1 over P450 1B1 enzymes. 6-PN was determined to be an AhR agonist and activated AhR signaling pathways. Its activity in AhR activation was also found to contribute to down-regulation of ER protein due to the AhR/ER pathway crosstalk, so 6-PN could potentially reduce breast cancer risk through both modulation of the estrogen chemical pathway and the hormonal pathway.

Future mechanistic studies could involve RNA sequencing with the treatment of individual bioactive compounds and botanical extracts to look at the effects and signaling cascades in cellular level.

In conclusion, certain botanicals can potentially reduce breast cancer risk through modulation of estrogen oxidative metabolism. Licorice extract GI and hops extract both exhibited beneficial activity in estrogen metabolism modulation. For women taking licorice supplements, GI would be a safer choice over GG and GU, since it provides both anti-inflammatory activity and down-regulation of estrogen oxidative metabolism. The differential effects seen from non-tumorigenic versus tumorigenic breast cell lines point out

the potential different effects of hops on healthy women and breast cancer patients. For healthy postmenopausal women, hops could help relieve postmenopausal symptoms through the actions of its estrogenic compound 8-PN, and prevent carcinogenesis through the actions of the chemopreventive agent xanthohumol. Additionally, hops could also potentially reduce the risk of breast cancer through up-regulation of estrogen 2-hydroxylation metabolism. However, hops might not be a top choice for women with breast cancer, since it also up-regulates the estrogen 4-hydroxylation pathway in tumorigenic breast cells.

LicA from licorice and 6-PN from hops were identified to have corresponding activities in posing beneficial effects on estrogen oxidative metabolism modulation. Thus, these bioactive compounds together with other previously determined bioactive compounds can be used as markers for botanical dietary supplement standardization. Since safety and efficacy for most of the botanical supplement products are not established due to lack of standardization, future clinical studies are in need with standardized botanicals targeting women in specific groups such as age, menopause status, cancer status, and etc. The metabolism and pharmacokinetics of botanicals will need to be determined to ensure the exposure of efficacious concentrations in clinical settings. These studies would ultimately help design and standardize botanical dietary supplements with multiple beneficial effects (efficacy), minimal adverse effects (safety), and reproducibility of biological activities (standardization).

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APPENDICES

APPENDIX A

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To: Shuai Wang <swang88@uic.edu>

Dear Mr. Wang,

Thank you very much for your patience. Please accept this email as confirmation that you may reuse your article content in your thesis.

Please let me know if you have any further questions.

Many thanks,
Maggie

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APPENDIX A (continued)

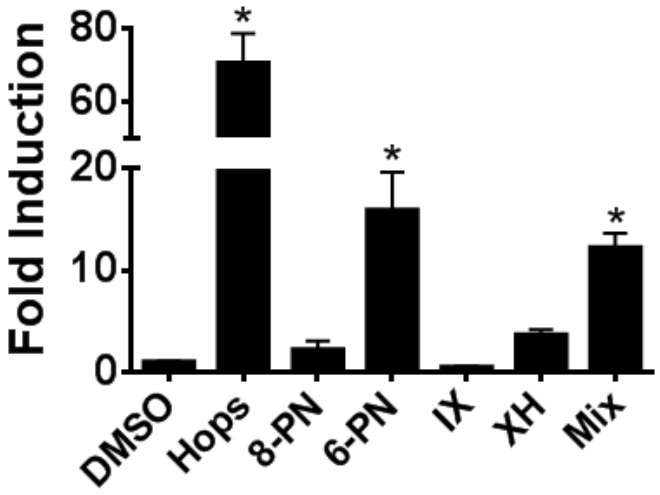


Figure S1. Hop compounds partially contribute to the activity of hops extract for up-regulation of P450 1A1/1B1 activity. MCF-7 cells were treated with hops extract (2.5 µg/ml) as well as the four marker hop compounds at the equivalent amount to those present in the extract, individually and as a mixture (6-PN 0.084 µM, 8-PN 0.024 µM, IX 0.070 µM, and XH 2.3 µM) for 48 h. Cells were then measured for EROD activity as previously described and the results were analyzed by one-way ANOVA for comparison of treatment groups with control, * $p < 0.05$.

APPENDIX A (continued)

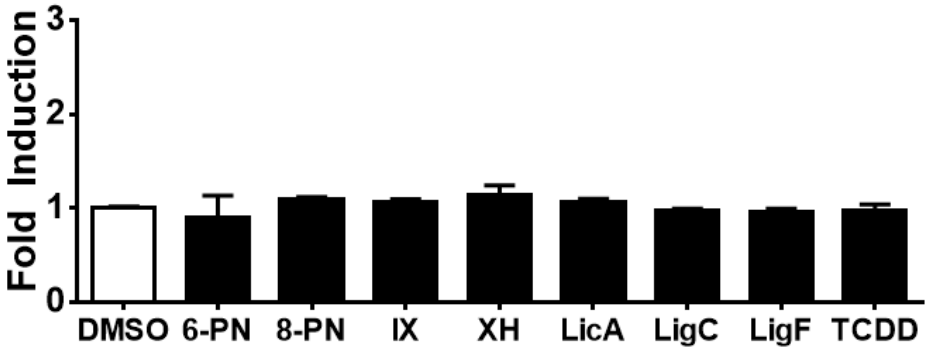


Figure S2. COMT mRNA expression was not affected by hop/licorice compounds in MCF-10A cells. COMT mRNA expression were analyzed after 24 h via qPCR in MCF-10A cells treated with DMSO control, 6-PN, 8-PN, IX, XH, LicA, LigC, LigF (1 µM), and TCDD (10 nM). Results are the means ± SEM of three independent experiments.

APPENDIX A (continued)

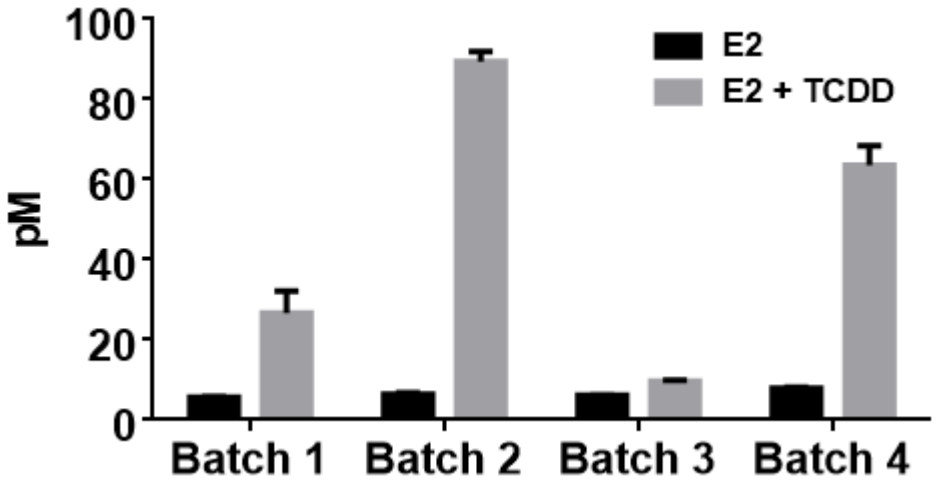
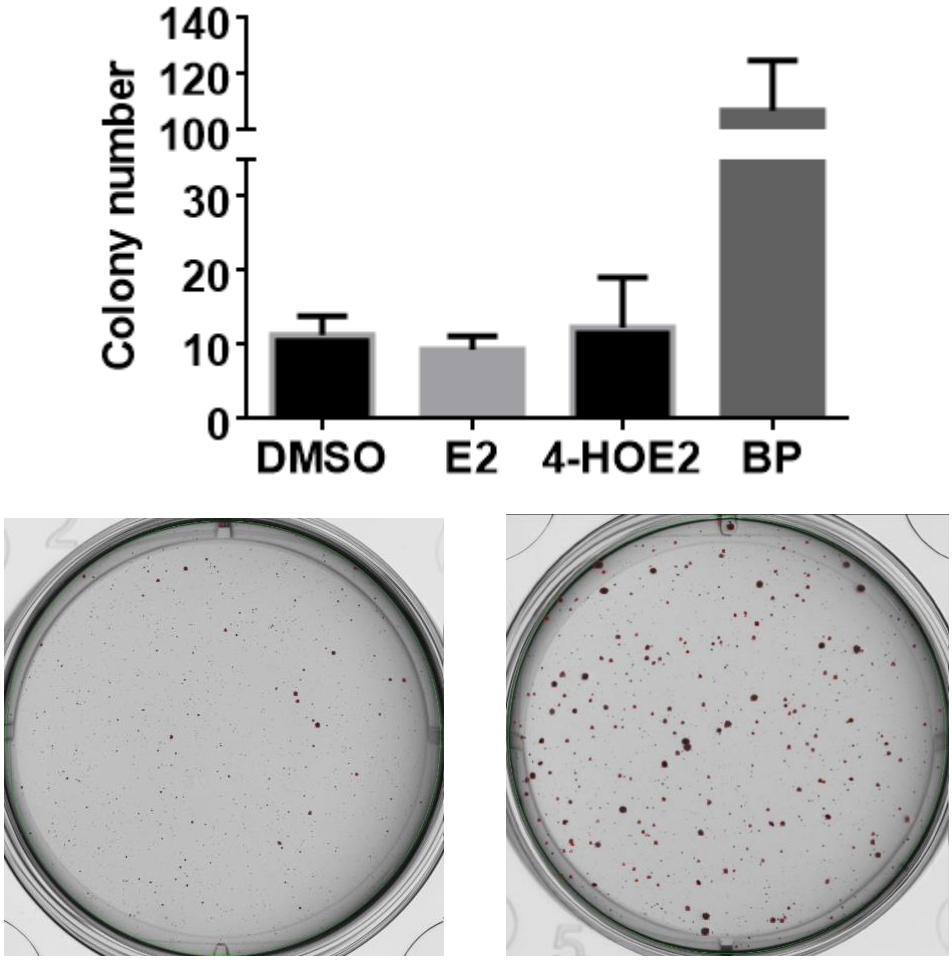


Figure S3. Differential induction of 4-MeOE$_1$ by TCDD with different batches of MCF-10A cells. Four different batches of MCF-10A cells were treated with estradiol (1 μM) and TCDD (10 nM) for two days and the amount of 4-MeOE$_1$ was quantified.

APPENDIX A (continued)



DMSO BP

Figure S4. MCF-10A malignant transformation. MCF-10A cells were treated with estradiol (E2), 4-hydroxyestradiol (4-HOE2), or benzo(*a*)pyrene (BP) at 1 μM for 5 weeks and cells were plated into soft agar for another 3 weeks. The colonies were counted with spherical diameter >200 μm.

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- Utilized hydrogel matrix drug administration to address drug solubility issue and conducted behavioral studies with Apolipoprotein E transgenic mice.
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- Evaluation of stability and DNA adduct formation kinetics/mechanisms with several ADC payloads.
- Discovered and applied a new depurination method to the study of DNA adducts.

Pfizer Global Research & Development Groton, CT

Biotransformation intern 05/2014 - 08/2014

-Evaluation of a micropatterned co-cultured (MPCC) hepatocyte system to predict preclinical and human specific drug metabolism

- MetID studies with drugs that have shown human specific metabolism through AO, CYP2C9, UGT1A4 and N-Acetyltransferase with LC-MS/MS.
- Metabolic profile comparison of drugs across species with rat, dog, monkey and human hepatocyte suspension and MPCC system.

-Assessment of qualitative and quantitative NMR in a drug metabolism environment.

- Performed metabolite fractionation and NMR analysis with structurally diverse drugs having differential Phase I/II metabolism.
- Utilized high resolution NMR to test its limit of structural elucidation and

quantification of samples purified from *in vitro* drug metabolism studies.

Publications:

1. **Wang S**, Dunlap TL, Howell CE, Mbachu OC, Rue EA, Chen SN, Pauli GF, Dietz BM, and Bolton JL. “Hop (*Humulus Lupulus L.*) extract and 6-prenylnaringenin induce P450 1A1 catalyzed estrogen 2-hydroxylation” *Chemical Research in Toxicology* (Epub 2016)

2. Ballard TE, **Wang S**, Cox LM, Moen MA, Krzyzewski S, Ukairo O, and Obach RS. “Application of a micropatterned co-cultured (MPCC) hepatocyte system to predict preclinical and human specific drug metabolism” *Drug Metabolism and Disposition* 44,172-179 (2016)

3. Dunlap TL, **Wang S**, Simmler C, Chen SN, Pauli GF, Dietz BM, and Bolton JL. “Differential effects of *glycyrrhiza* species on genotoxic estrogen metabolism: licochalcone A downregulates P450 1B1, whereas isoliquiritigenin stimulates it” *Chemical Research in Toxicology* 28(8), 1584-1594 (2015)

4. Hemachandra M, Patel HK, Esala P, Choi J, **Wang S**, Wang Y, Thayer E, Scism R, Michalsen B, Xiong R, Siklos M, Bolton JL, and Thatcher GRJ. “SERMs attenuate estrogen-induced malignant transformation of human mammary epithelial cells by upregulating detoxification of oxidative metabolites” *Cancer Prevention Research* 7(5), 505-515 (2014)

5. Yu M, Jiao L, and **Wang S**, Jiao Y. “Determination of Chlorphenamine Maleate in Bitai Tablets by HPLC” *China Pharmacist* 13(10), 1530-1531 (2010)

6. Han M, Liu X, Huang Q, and **Wang S.** “Establishment of Quality Standard on Xinnaokang Capsule” *China Pharmacist* 13(6), 905-908 (2010)

Oral/poster presentations:

Wang S, Dunlap TL, Howell CE, Mbachu OC, Rue EA, Chen SN, Pauli GF, Dietz BM, and Bolton JL. “Hop (*Humulus Lupulus L.*) extract and 6-prenylnaringenin induce P450 1A1 catalyzed estrogen 2-hydroxylation” *54th annual MIKI conference*, Iowa City, IA (2016)

Wang S, Dunlap TL, Simmler C, Chen SN, Pauli GF, Dietz BM, and Bolton JL. “Licochalcone A from *Glycyrrhiza Inflata* inhibits P450 1B1 mediated estrogen chemical carcinogenesis in MCF-10A cells” *53rd annual MIKI conference*, Lawrence, KS (2015)

Wang S, Snelten C, Ramos RF, Chen SN, Pauli GF, Dietz BM, and Bolton JL. “Bioactive compounds in hops (*humulus lupulus l.*) differentially modulate oxidative estrogen metabolism: isoxanthohumol correlates with chemopreventive activity” *53rd annual SOT conference*, Phoenix, AZ (2014)

Wang S, Luo J, Qin Z, Xiong R, Thayer E, and Thatcher GRJ. “Validation and development of ApoE targeted treatment for Alzheimer’s disease by RXR agonists” *51st annual MIKI conference*, Minneapolis, MN (2013)

Piyankarage SC, Wang YT, **Wang S**, and Thatcher GRJ. “A mass spectrometry-based approach for mapping and quantitation of both nitrosation and oxidation of the cysteome” *61st ASMS Conference*, Minneapolis, MN (2013)

Honors and Awards:

W.E. van Doren Scholar, UIC	2016
Poster Award, College of Pharmacy Research Day, UIC	2015
Teaching Certificate, College of Pharmacy, UIC	2012
Academic Excellence Scholarship, China Pharmaceutical Univ.	2007 - 2010
Honor of Excellent Class Leader, China Pharmaceutical Univ.	2008 - 2010
Undergraduate Scholarship by Suzhou Capsugel (Pfizer Inc.)	2009 - 2011

Professional Affiliations:

Society of Toxicology

The American Association for the Advancement of Science