The Role of Estradiol in the Modulation of Estrogen Chemical Carcinogenesis by Isoflavones

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DISSERTATION

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To my parents Robert and Neppie for encouraging my curiosity to explore the world around me and to my late advisor Judy Bolton without whom this would not have been written.

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LIST OF ABBREVATIONS

AhR	Aryl hydrocarbon receptor
ARNT	Aryl hydrocarbon receptor nuclear translocator
BA	Biochanin A
BDS	Botanical dietary supplements
COMT	Catechol-O-methyl transferase
CYP	Cytochrome P450 gene
DZ	Daidzein
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E_1	Estrone
E_2	17β-Estradiol
ER	Estrogen receptor
EROD	Ethoxyresorufin O deethylase
FBS	Fetal Bovine Serum
FN	Formononetin
GG	*Glycyrrhiza glabra*
GI	*Glycyrrhiza inflata*
GN	Genistein
GU	*Glycyrrhiza uralensis*
GSH	Glutathione
GST	Glutathione S transferase
HER2	Human epidermal growth factor receptor 2

LIST OF ABBREVATIONS (continued)

HPLC	High-performance liquid chromatography
HT	Hormone replacement therapy
IX	Isoxanthohumol
LicA	Licochalcone A
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
P450	Cytochrome P450 gene
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
RCE	Red clover extract
ROS	Reactive oxygen species
RNA	Ribonucleic acid
SB	Soy bean extract
SD	Standard deviation

LIST OF ABBREVATIONS (continued)

SEM	Standard error of the mean
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

Women may spend the last third of their lives in post menopause. The transition to menopause occurs when ovaries stop producing estrogens resulting in a significant drop in circulating levels of these hormones. This drastic decline in levels of circulating estrogens leads to the rise of uncomfortable symptoms, such as hot flashes, night sweats, mood swings, insomnia, vaginal dryness, and osteoporosis.

Although circulating estrogens are low in postmenopausal women, the local production of estrogens in organs such as breast is not significantly compromised. In the fibroblasts of adipose tissues in the breast, estrogens, mainly the interconvertible estradiol (E_2) and estrone (E_1), are converted by aromatase (P450 19A1) from testosterone and androstenedione, creating an estrogen-rich local environment comparable to that in premenopausal women.

The treatment of choice for the alleviation of uncomfortable menopausal symptoms caused by these low circulating levels hormone levels was hormone therapy (HT): the supplementation of hormones with estrogen or estrogen + progestin. That began to change in May 2002 when the estrogen + progestin arm of the large-scale Women's Health Initiative (WHI) was halted more than three years early because of an increased risk of invasive breast cancer development in otherwise healthy menopausal women. Although there were several important points lost in the media wave that followed these concerning results, the findings raised major concerns about the safety of HT and prompted women to seek alternative options to relieve menopausal ailments.

Largely because of these concerns many women turn to natural products as an alternative to hormone therapy. Botanical dietary supplements (BDS) are a popular

choice because of their long history of use in traditional medicine and the common perception of them as “safe” because they are natural. Isoflavone containing supplements derive much of their popularity because Asian women, who traditionally have a high-isoflavone diet, report fewer and less severe hot flashes. Many isoflavones show estrogenic properties that may alleviate menopausal symptoms by binding to estrogen receptors (ERs), although their efficacy in humans is controversial. Moreover, they can have chemoprevention effects by mediated pathways such as estrogen oxidative metabolism. However, estrogenicity, chemopreventive properties, and safety of BDS are not fully understood.

Breast cancer is the most commonly diagnosed cancer in women in the United States with an estimated 13% lifetime risk of diagnosis. Breast cancer risk is correlated with length of time of exposure to estrogens, particularly estradiol (E_2). Estrogen carcinogenesis can be broken down into at least three mechanisms: hormonal, chemical, and epigenetic. In the hormonal pathway, E_2 can bind to ERα, promoting cell proliferation. In the chemical pathway, the aryl hydrocarbon receptor (AhR) mediates induction of oxidative estrogen metabolizing enzymes, P450 1A1 and P450 1B1. Metabolism by P450 1B1 can lead to the formation of DNA adducts, while metabolism through P450 1A1 is associated with decreased cancer risk. In the epigenetic pathway, ligands to ERα can promote methylation of the promoter region of *CYP1A1*, the gene encoding for P450 1A1. This methylation selectively suppresses expression of this “detoxification” enzyme. This project primarily focuses on the combined effects of the

chemical and epigenetic pathways and the modulation of these pathways by isoflavones in BDS consumed by women for menopausal symptom relief.

Extracts can vary dramatically in chemical composition, and the chemical composition of the extract often differs markedly from the form of the active compounds in the body. For instance, soy and red clover extracts often contain isoflavones in their inactive glycosylated form. The glycosylated isoflavone undergoes deglycosylation to its form the active aglycone in the intestine where it is absorbed. In the liver, these aglycones undergo phase II metabolism, primarily glucuronidation, by glucuronyltransferases (UGTs). The aglycone form produces the biological effect. Thus, it may be necessary to hydrolyze an extract to expose its biological activity *in vitro*.

This study was primarily conducted with two isoflavone rich extracts, a hydrolyzed standardized red clover extract (RCE) and a hydrolyzed soy bean extract (SB), and their major bioactive isoflavones. RCE is a standardized hydrolyzed extract studied for more than a decade in the UIC/NIH Center for Botanical Dietary Supplements Research. As part of this study we investigated the effects of hydrolysis on a soy bean extract and chose one of these extracts for further study. Ingestion of these extracts like those studied results in the presence of similar bioactive estrogenic isoflavones, genistein (GN) and daidzein (DZ) in the body. RCE contains both ER agonists, GN and DZ, and AhR agonists biochanin A (BA) and formononetin (FN). In the body, the AhR agonists in red clover, BA and FN are metabolized in the liver and other tissues into compounds with primarily estrogenic activity, GN and DZ. The unhydrolyzed soy beans extract (SB),

contains primarily ER agonists, GN and DZ in their glycosylated forms, genistin and daidzin, respectively. These are deglycosylated in the body into estrogenic GN and DZ. Since the hydrolyzed RCE contains AhR agonists, it can serve as a BDS model to study the effects AhR and ER agonists combined, while hydrolyzed soy extract can serve to investigate primarily estrogenic effects.

Estrogenic isoflavones (phytoestrogens) act on ERα, the same receptor as the endogenous estrogens present in women's breast tissue. This estrogen receptor is profoundly important in both cancer risk and, more specifically, estrogen chemical carcinogenesis through oxidative estrogen metabolism. However, the role of E_2 in the modulation of estrogen chemical carcinogenesis by isoflavones has, to our knowledge, not been systematically studied. Thus, in this project we **hypothesized** that **the presence of estrogens in breast tissue significantly affects modulation of estrogen oxidative metabolism by isoflavones.**

To investigate this question, this study takes the approach of first mechanistically examining these effects without the presence of E_2, then creating three conditions (no, low, and high E_2) in the same cell line and comparing the changes in metabolism within each system to those in the other systems. However, this approach required the creation of a new activity assay to distinguish between the ER-mediated and AhR-mediated effects on P450 1A1/1B1 activity. The LC-MS/MS assay measuring the $MeOE_1$ metabolites of E_1/E_2 used of this purpose in chapter 3 requires the presence of a high dose of E_2, therefore, could not reliably measure the changes in activity in an estrogen free or low estrogen environment.

To investigate this question, this study takes the approach of first mechanistically examining these effects without the presence of E_2, then creating three conditions (no, low, and high E_2) in the same cell line and comparing the changes in metabolism within each system to those in the other systems. However, this approach required the creation of a new activity assay to distinguish between the ER-mediated and AhR-mediated effects on P450 1A1/1B1 activity. The LC-MS/MS assay measuring the $MeOE_1$ metabolites of E_1/E_2 used in chapter 3 requires the presence of a high dose of E_2, therefore, could not reliably measure the changes in activity in an estrogen free or low estrogen environment.

To gain a baseline for studying the effects of AhR agonist + ER agonist isoflavones on estrogen oxidative metabolism in cell-based models with no E_2, a red clover extract (RCE) was studied along different points in the pathways known to affect estrogen oxidative metabolism (Figure 2).

The effect of the extract (RCE) on ERα+ MCF-7 WS8 cells was a selective induction of the genotoxic P450 1B1 4-hydroxylation pathway at high concentration and a selective suppression of the P450 1A1 pathway in lower concentrations. In these cells, the ER-agonists GN and DZ selectively suppressed the P450 1A1 pathway. Cells treated with BA and FN partially metabolized those AhR agonists in GN and DZ, respectively. BA and FN treated cells were found to modulate P450 1A1/1B1 in a manner similar to the overall RCE extract. In an ER- non-tumorigenic cell line, little change occurred with treatment. These results suggested that RCE may be safe in tissue that does not contain ERα. However, the estrogen mediated effects may be of

concern in ERα+ tissues due to the ability to either suppress the P450 1A1 (detoxification) pathway in the absence of an AhR agonists or to selectively induce the P450 1B1 (genotoxic) pathway in the presence of them.

Since ERα is only involved in the epigenetic regulation of oxidative estrogen metabolism, the question of the effects of the presence of E_2 was simplified by only including estrogenic isoflavones and not AhR agonists. However, the model-based approach required the creation of a new activity assay to measure the ER mediated and AhR mediated effects on P450 1A1/1B1 activity. The new assay incorporated spheroids and a P450 1B1 inhibitor into the already existing EROD assay.

This work was performed in the UIC/NIH Center for Botanical Dietary Supplements Research that focuses on studying botanical extracts used for women's health. The newly developed 3D-EROD assay was used in conjunction with computational modeling and an alkaline phosphatase assay of estrogen activity to investigate existing hypotheses and form new ones about a variety of botanical extracts and bioactive compounds used for menopausal women's health. Roseroot (*Rhodiola rosea*), Milk thistle (*Silybum marianum*), and dong quai, the dried root of *Angelica sinensis* were investigated for biological activity using combinations of these techniques due to their use as women's health supplements. Through this evaluation we determined that a 4 day autohydrolyzed soy bean extract that demonstrated high estrogenic activity and did not show signs of AhR mediated induction would be used in the final part of this study. Through these evaluations, fractions high in fatty acids were found to act as probable AhR agonists, and hypotheses were formed about compounds

found in botanical extracts that should to be further studied in future structure activity relations studies.

Although concentrations of E_2 in breast tissue do not correlate well with serum levels, the pM to nM range is considered to be biologically relevant in breast tissue. The estrogen-mediated suppression of P450 1A1 was dose related in the pM to nM range of E_2 treatment. A dose on the low end of the curve (1 pM) was chosen as the low E_2 model, and a dose on the high end of the curve (1 nM) as was used for the high E_2 model. As expected, with no E_2, the estrogenic extract and major bioactive compounds (GN, DZ) were found to selectively suppress P450 1A activity. Simply co-treating with E_2 (1 nM) did not change this effect. However, combined pre- and co-treatment with E_2 reversed the ERα-mediated P450 1A1 suppression by the estrogenic isoflavones. The P450 1A1 suppression slightly decreased in response to the low dose (1 pM) and was reversed at the high dose of E_2 (1 nM) model when compared to the same isoflavone/extract treatment without E_2 co-treatment. These findings agreed with data from animal and clinical studies of the effects of estrogenic isoflavones on estrogen metabolism in the literature.

Our findings suggest that, contrary to traditional estrogen free *in vitro* models, in models with high levels of estrogens, suppression of the detoxifying P450 1A1 pathway by phytoestrogens does not occur. These data suggest that the suppression of P450 1A1 by estrogenic isoflavones might not be a concern in women. However, more mechanistic studied, *in vivo*, and clinical studies are warranted to analyze if these results are translatable to women. These results also emphasize an increasing need for

biologically relevant *in vitro* models to better understand biological effects before *in vivo* or clinical studies.

1 INTRODUCTION

1.1 Menopause:

Women may spend the last third of their lives in post menopause (Minino 2011). The transition to menopause occurs when ovaries stop producing estrogens, resulting in a significant drop in circulating levels of these hormones (Aidelsburger et al. 2012). This drastic decline in levels of circulating estrogens leads to the rise of uncomfortable symptoms, such as hot flashes, night sweats, mood swings, insomnia, and vaginal dryness, and osteoporosis (Roush 2012, Ortmann and Lattrich 2012).

However, although circulating estrogens are low in postmenopausal women, the local production of estrogen in organs such as breast is not significantly compromised. In the fibroblasts of adipose tissues in breast, estrogens, mainly estradiol (E_2) and estrone (E_1), are converted by aromatase (P450 19A1) from testosterone and androstenedione (Sasano, Miki, et al. 2009, Sasano, Nagasaki, et al. 2009), creating an estrogen-rich local environment (van Landeghem et al. 1985) comparable to that in premenopausal women (Yaghjyan and Colditz 2011).

1.2 Women's Health Initiative:

The first line of treatment for the alleviation of uncomfortable menopausal symptoms initiated by low circulating estrogen levels was hormone therapy (HT): the supplementation of hormones with estrogen or estrogen + progestin. That changed in May 2002, when the estrogen + progestin arm of the large scale Women's Health Initiative (WHI) was halted more than three years early because of an increased risk of invasive breast cancer development in otherwise healthy menopausal women

(Rossouw et al. 2002b). Although there were several important points lost in the media wave that followed these concerning results (Rossouw et al. 2007, Lenfant et al. 2011), the findings raised major concerns about the safety of HT and prompted women to seek alternative options to relieve menopausal ailments.

1.3 Botanical dietary supplements for women's health

Largely because of these concerns many women turn to natural products as an alternative to hormone therapy. Botanical dietary supplements (BDS) are a popular choice because of their long history of use in traditional medicine and the common perception of them as "safe" because they are natural (Poluzzi et al. 2014, Geller and Studee 2005, Pitkin 2012, Wang et al. 2011). As of 2017, the sales of botanical dietary supplements, including those used to address symptoms of menopause, reached $8.085 billion in the United States, an 8.5% increase from the previous year (Smith 2018). Isoflavone containing supplements, in particular, derive much of their popularity because Asian women, who have a high-isoflavone diet, report fewer and less severe hot flashes (Taechakraichana et al. 2002). Some isoflavones-are estrogenic and may alleviate symptoms by binding to estrogen receptors, although their efficacy in humans is controversial (Hajirahimkhan, Dietz, and Bolton 2013a, Poluzzi et al. 2014, Mintziori et al. 2015). Moreover, they can affect chemoprevention pathways by modulating P450 1A1/1B1 metabolism. However, estrogenicity, chemopreventive properties, and safety of BDS are not fully understood (Mintziori et al. 2015).

1.4 Breast cancer:

Breast cancer is the most commonly diagnosed malignancy in women in the United States with an estimated one in eight women developing breast cancer in her lifetime (Siegel, Miller, and Jemal 2016a). Breast cancer risk is correlated with length of time of exposure to estrogens, particularly E_2 (2002). There is a 5% reduction in risk for each year of delayed onset of menses (Hunter et al. 1997), and risk increases with later onset of menopause (1997). Moreover, long-term risk decreases with pregnancy (2002). During pregnancy, there is a large increase in estriol (E_3) (Musey et al. 1987), an estrogen with significantly less affinity for the estrogen receptor than E_2 and which is not known to be metabolized into the 4-hydroxylated genotoxic quinones formed by estrone (E_1) or E_2 (Cavalieri and Rogan 2006).

1.4.1 **Mechanisms of estrogen mediated cancer risk**

Estrogen carcinogenesis can be broken down into at least three mechanisms: hormonal, chemical, and epigenetic. In the hormonal pathway, E_2 can bind to ERα promoting cell proliferation (Sotoca et al. 2008). In the chemical pathway, oxidative estrogen metabolism by P450 1B1 can lead to the formation of DNA adducts (Bolton and Thatcher 2007). In the epigenetic pathway, binding to ERα can induce methylation of the promoter region of *CYP1A1*, the gene encoding for P450 1A1. This methylation selectively suppresses the expression of this “detoxification” enzyme. This project primarily focuses on the combined effects of the chemical and epigenetic pathways. (Figure 2) and the modulation of these pathways by isoflavones in BDS consumed by women for menopausal symptom relief.

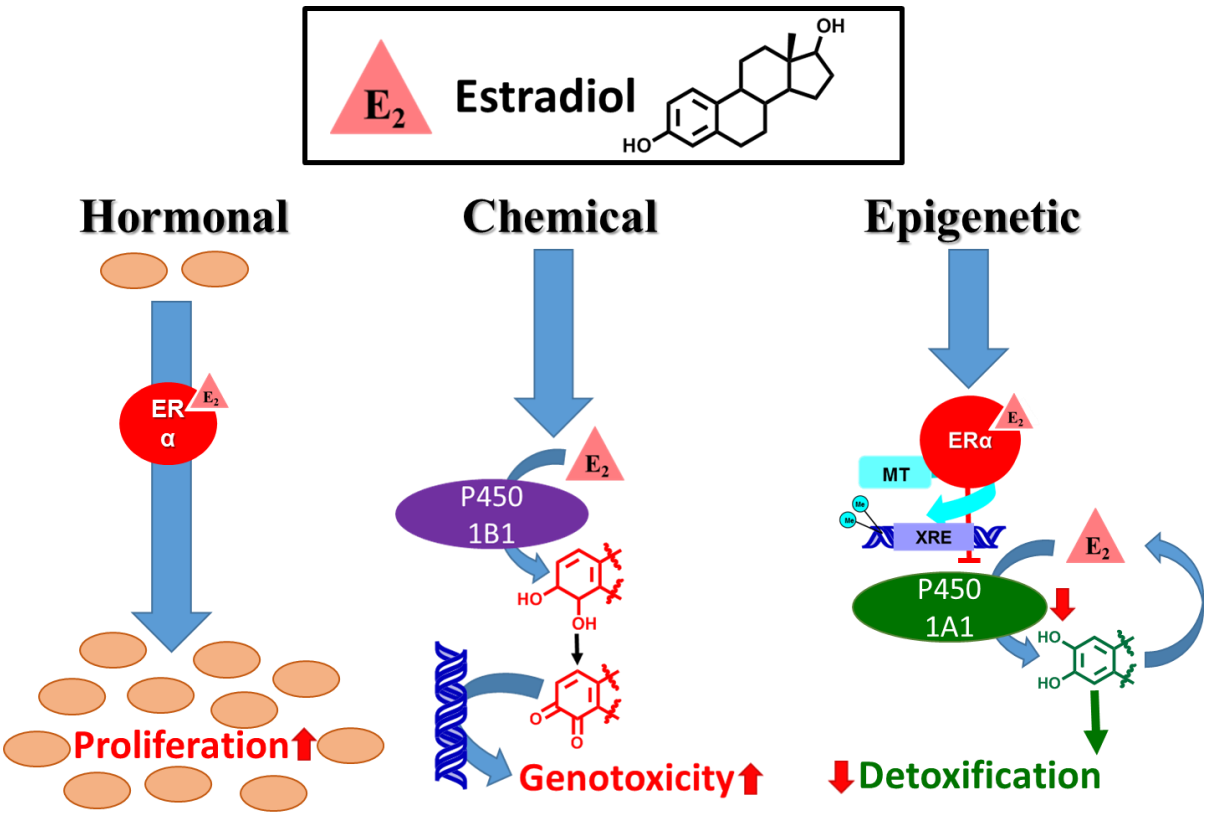

Figure 1: Three pathways of estrogen carcinogenesis:
Estradiol binds to ERα and promotes cell proliferation. aryl hydrocarbon receptor (AhR), a mediator of estrogen oxidative metabolism, which can ultimately lead to the formation of genotoxic DNA adducts. ERα binding leads to methylation of the promotor region epigenetic effect by selectively suppressing the “detoxifying” enzyme, P450 1A1 (epigenetic).

1.4.1.1 Hormonal pathway

ERα binding leads to dissociation of chaperone proteins and dimerization formation with another ERα protein. This ligand bound ERα dimer translocates into the nucleus where it binds to the estrogen response element (ERE) and co-activators are recruited. This receptor DNA complex initiates ERα targeted gene transcription (Yager and Davidson 2006b), including genes that promote cell proliferation (Beatson 1983, Rossouw et al. 2002a). ERβ selective agonists inhibit cell proliferation (Helguero et al. 2005). Isoflavones can bind to ERα and/or ERβ, with ERβ preferential agonists exerting a concentration-dependent antiproliferation effect (Zhao et al. 2015).

1.4.1.2 Chemical pathway

In breast tissue, E_2 and E_1 are metabolized by P450 1B1 into their 4-hydroxylated metabolites or by P450 1A1 into their 2-hydroxylated metabolites (Cavalieri and Rogan 2006, Bolton and Dunlap 2017a, Moore et al. 2016, Falk et al. 2013, Fuhrman et al. 2012, Dallal et al. 2014, Ziegler et al. 2015) (Figure 2). P450 1B1 is associated with carcinogenesis because it is overexpressed in malignant tissues (Wen et al. 2007). P450 1B1 produces 4-OHE_1/E_2 which are oxidized by peroxidases/P450s to genotoxic quinones (4-OHE_1/E_2-Q) that oxidize and alkylate DNA and generate depurinating adducts (Cavalieri and Rogan 2016a). On the other hand, the 2-hydroxylation pathway can be considered detoxifying because of the decreased risk of cancer observed in women with higher 2-hydroxylation metabolites. Metabolism by P450s 1A1 and 1B1 is classically regulated by the aryl hydrocarbon Receptor (AhR), which, when activated, upregulates both P450s. (Figure 2)

Further, the concentration of estrogen quinones can be diminished though methylation of catechol estrogens catalyzed by catechol-O-methyl transferase (COMT) (Figure 2), reduction of estrogen quinones to catechol estrogens by NAD(P)H–quinone oxidoreductase 1 (NQO1) (Cavalieri and Rogan 2016a, Lu et al. 2008), as well as conjugation of estrogen quinones to glutathione catalyzed by glutathione S-transferases (GST) (Yang et al. 2013). Isoflavones can act on any or multiples of the above enzymes to modulate this pathway. Isoflavones can act on any or multiples of the above enzymes to modulate this pathway. Of these enzymes, this project focuses primarily on AhR-mediated induction/suppression of P450 1A1 and 1B1 expression and direct inhibition of P450 1A1 and/or P450 1B1 activity as well as the epigenetic pathway below.

1.4.1.3 Epigenetic pathway:

Epigenetic pathways contribute to estrogen carcinogenesis. In one of these, E_2 activates ERα which following dimerization binds to AhR/ARNT on XREs in the *CYP1A1* promoter which causes methylation resulting in repression of CYP 1A1 expression and these are methylated (Ramos Alvarenga et al. 2014, Marques, Laflamme, and Gaudreau 2013). This mechanism specifically downregulates *CYP1A1* without affecting *CYP1B1* expression in MCF-7 cells (Marques, Laflamme, and Gaudreau 2013). (Figure 2) As mentioned above, isoflavones can bind to ERα, activating this pathway.

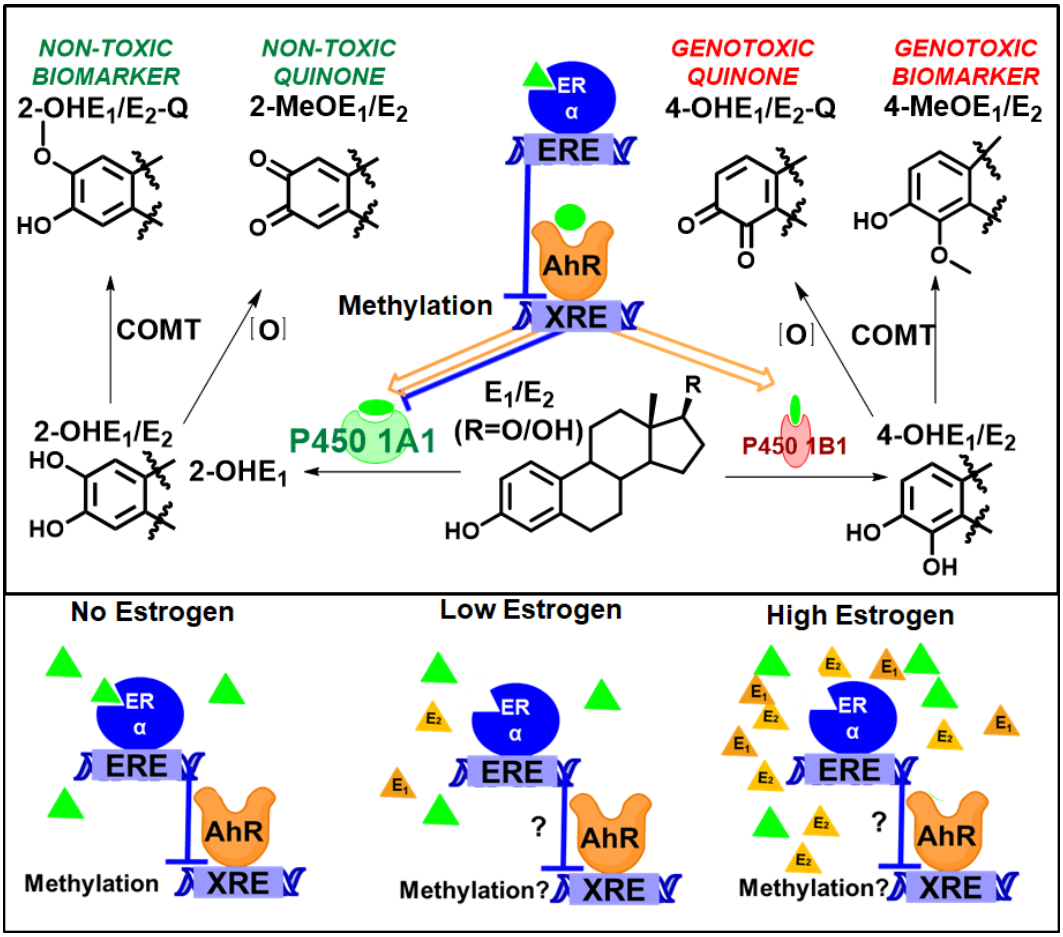

Figure 2: Combined effects of the chemical and epigenetic effects on estrogen oxidative metabolism with and without E_2.
AhR activation induces both the non-toxic P450 1A1 pathway and the genotoxic P450 1B1 pathway. ERα activation selectively suppresses the P450 1A pathway. Isoflavones can bind to ERα, AhR or directly inhibit P450 1A1 and/or P450 1B1. The effect of E_2 on the ERα mediated effects in this system are investigated using three model systems: no estrogen, low estrogen, and high estrogen.

1.5 Estrogen metabolites and cancer risk in women

Although parent estrogen levels linked to the hormonal pathway have the strongest associations with breast cancer risk in both low risk Asian (Moore et al. 2016) and high risk (western) countries (Falk et al. 2013, Fuhrman et al. 2012, Dallal et al. 2014, Ziegler et al. 2015), there is an inverse relationship between breast cancer risk and 2-hydroxylation of parent estrogens (E_2 and E_1) found in both serum and urine measurements of postmenopausal women (Falk et al. 2013, Fuhrman et al. 2012, Dallal et al. 2014, Ziegler et al. 2015).These studies include both prospective and retrospective nested, case-controlled studies and case-cohort studies. This link was not observed in premenopausal women (Morimoto et al. 2012).

The link between 4-hydroxylation and breast cancer risk is less clear with some studies finding inverse relationships between the 4-hydroxylation pathway catechol estrogens and postmenopausal breast cancer risk (Dallal et al. 2014), but most findings show no significant correlation between 4-hydroxylation metabolites and cancer risk. In short, although evidence of the link between 4-hydroxylation and breast cancer risk in women is compelling, the beneficial effects of an induction in 2-hydroxylation pathway is most supported by studies in women.

1.6 Extract composition:

Isoflavones are widely consumed in Asian countries in the form of soy, although soy food products are increasing in popularity in western countries (He and Chen 2013). More recently, because, in part, Asian women report fewer and less severe hot flashes (Taechakraichana et al. 2002), many women have turned to isoflavone containing

extracts such as soy and red clover for menopausal symptom relief (Touillaud et al. 2019).

1.6.1 **Isoflavones: Human metabolism**

Extracts can vary dramatically in chemical composition (Uifălean et al. 2015), and the chemical composition of the extract often differs markedly from the form of the active compounds in the body upon ingestion. For instance, soy extracts often contain phytoestrogens in their estrogenically inactive glycosylated form. The glycosylated isoflavone undergoes deglycosylation to its form the active aglycone in the intestine where it is absorbed. In the liver, these aglycones undergo phase II metabolism, primarily glucuronidation, by glucuronyltransferases (UGTs) (Pritchett et al. 2008, Bolca et al. 2010). Since the aglycone is responsible for the biological effects in tissues, it may be necessary to hydrolyze an extract to expose its biological activity *in vitro*.

This study was primarily conducted with two isoflavone rich extracts, red clover extract (RCE) and soy bean extract (SB) and their major bioactive isoflavones. Ingestion of these extracts results in the presence of similar estrogenic isoflavones, GN and DZ, in the body. However, their biological effects *in vitro* vary because one of these extracts, red clover extract (RCE) contains both ER agonists, GN and DZ, and AhR agonists, BA and FN, while the other, soy bean extract (SB), contains primarily ER agonists, GN and DZ in their glycosylated forms genistin and daidzin, respectively. However, upon ingestion in the body, the AhR agonists in red clover, BA and FN are metabolized into compounds with primarily estrogenic activity, GN and DZ (Roberts et al. 2004). Thus, using RCE and a hydrolyzed SB allows for the study of both the combined effects of the

chemical and epigenetic pathways as well as just the epigenetic pathway within the confines of BDS in vitro (Figure 3).

Estrogenic isoflavones act on ERα, the same receptor to which the endogenous estrogens present in women's breast tissue bind. This ER is profoundly important in both cancer risk and, more specifically, oxidative estrogen metabolism. However, the role of E_2 on the modulation of estrogen chemical carcinogenesis by isoflavones has, to our knowledge not been systematically studied. The **hypothesis** of this project is that **the presence of estrogens in breast tissue significantly affects modulation of oxidative estrogen metabolism by isoflavones.**

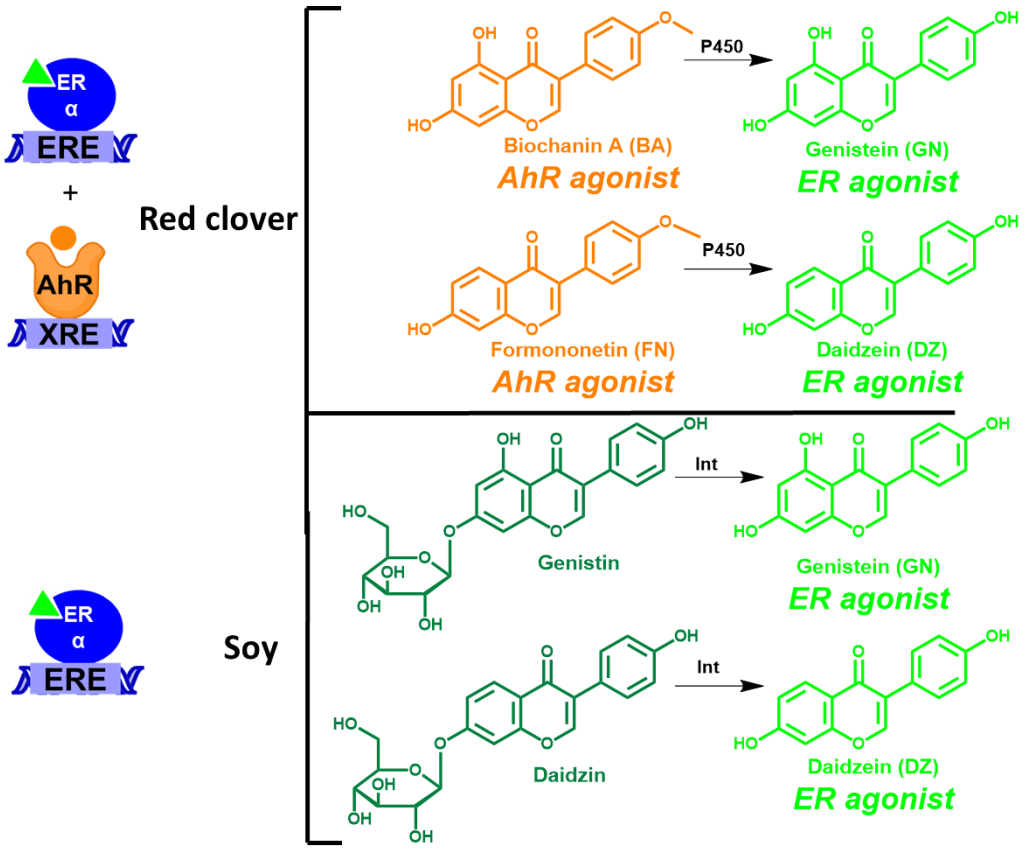

Figure 3: Major isoflavones in red clover and soy: The major bioactive isoflavones in red clover extract, BA and FN, are AhR agonists.
These metabolize both in the liver and extrahepatically in the breast are metabolized to ER agonists GN and DZ. Red clover has combined ER and AhR mediated effects

1.7 Approach and Complications

To investigate this question, this study takes the approach of first mechanistically studying these effects without the presence of E_2, then utilizing 3 systems (no, low, and high E_2) in the same cell line and comparing the changes in metabolism within each system to those in the other systems. However, this approach required the creation of a new activity assay to measure the ER-mediated and AhR mediated effects on P450 1A1/1B1 activity. The LC-MS/MS assay determining the methoxyestrone ($MeOE_1$) metabolites of E_1/E_2 used in Chapter 3 requires the presence of a high dose of E_2, therefore; it could not reliably used to measure the changes in activity in an estrogen free or low estrogen environment.

An assay not requiring the presence of estrogens, the 7-ethoxy-resorufin-O-deethylase (EROD) assay, detects AhR-mediated changes in P450 1A1 activity by measuring the conversion of 7-ethoxyresorufin-*O*-deethylase by the P450 1 class of enzymes into the fluorescent resorufin in a kinetic in-cell assay (Dunlap et al. 2015a, Wang et al. 2016a). However, this assay was limited in that ERα-mediated P450 1A1 suppression could not be detected and the effects of ER-agonists could not be determined in MCF-7 WS8 cells (chapter 4). This EROD assay was optimized to evaluate overall changes in ERα-mediated suppression and AhR-mediated induction/suppression in an assay adaptable to high-throughput screening. This assay was an essential tool to screen many compounds/extracts and to investigate the role of estradiol on the modulation of estrogen chemical carcinogenesis/estrogen oxidative metabolism by isoflavones

1.8 Specific Aims for this Study:

Aim 1: Investigate how ER and AhR agonistic isoflavones affect P450 1A1/1B1 metabolism of estrogens in the absence of E_2.

Aim 2: Create tools to measure P450 1A1/1B1 activity that can be used to study extracts in model systems containing different levels of E_2.

Aim 3: Establish whether isoflavones differentially modulate oxidative estrogen metabolism in models containing E_2 than in models without E_2.

2 METHODS

2.1 Chemicals, Extracts, and Reagents

All chemicals and reagents were purchased from Fisher Scientific 126 (Hanover Park, IL) or Sigma (St. Louis, MO), unless indicated below.

2.1.1 Red Clover Extract:

The RCE used in this study was a hydroalcoholic autohydrolyzed extract of the aerial parts of *Trifolium pratense L., Fabaceae*, which had been previously used in clinical trial (Booth et al. 2006, Geller et al. 2009).The extract contained 30% w/w isoflavones [BA (14.47%), FN (14.26%), GN (0.41%), and DZ (0.23%)] and was manufactured by PureWorld Botanicals, Inc. (South Hackensack, NJ) as described previously.

2.1.2 Soy Bean Extract:

Soybean, *Glycine max* (*g. max*) powder (10 g) was mixed with 250 mL of water. This mixture was kept in the incubator (25 °C) for 0, 2, 4, or 6 days. Samples were dried, defatted with hexanes (150 mL), and extracted with EtOH (150 mL). HPLC-UV was used to qualitatively assess the efficacy of auto-hydrolysis. Independent qHNMR quantitation used high-accuracy external calibration. These extracts were investigated for influence on P450 1A1/1B1 activity and estrogenicity in chapter 5 and only the 4-day hydrolyzed extract was studied in chapter 6.

2.2 Cell Lines and Culture Conditions.

2.2.1 Authentication:

All cell lines were authenticated via determination of the short tandem repeat (STR) profile using an ABI 3730xl DNA Analyzer and the Promega GenPrint 10 system (Promega, Madison, WI, USA) and GeneMapper 5.0 analysis software (Thermo Fisher Scientific, Waltham, MA, USA). HC-04 cells and MCF-10A cells were in 100% agreement with the STR profile according to the ATCC database. The MCF-7 WS8 cells were in 93% agreement with the MCF-7 cells from ATCC, however, they showed one allele deletion (D5S818:12) indicating a slight difference of the subclone MCF-7 WS8 cell line to the MCF-7 ATCC cell line (Jiang et al. 1992).

2.2.2 HC-04:

HC-O4 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). HC-04 cells were maintained in DMEM/F12 with 10% FBS and 1% penicillin–streptomycin. HC-04 cells, which are nearly identical to HepG2 cells (BEI Resources, Manassas, VA and ATCC database), were maintained in DMEM/F12 with 10% FBS and 1% penicillin–streptomycin.

2.2.3 MCF-10A:

Cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). MCF-10A cells were cultured in DMEM/F12 supplemented with epidermal growth factor (20 ng/mL), cholera toxin (100 ng/mL), hydrocortisone (0.5 μg/mL), insulin (10 μg/mL), 5% horse serum, and 1% penicillin−streptomycin.

2.2.4 **Ishikawa:**

The ERα endometrial carcinoma cells (Ishikawa) were provided by Dr. R. B. Hochberg (Yale University, New Haven, CT, USA) and were maintained in Dulbecco’s

modified Eagle's medium (DMEM/F12) containing 1% sodium pyruvate, 1% nonessential amino acids (NEAA), 1% Glutamax-1, 0.05% insulin, and 10% heat-inactivated fetal bovine serum (FBS) as described previously (Hajirahimkhan et al. 2013). An estrogen-free medium was prepared similarly but by using phenol-red-free medium and 10% charcoal-stripped FBS.

2.2.5 **MCF-7 WS8 cells:**

Cells were provided by Dr. C. Jordan . They are an estrogen sensitive cell line (ER+) and were cloned from MCF-7 cells, as previously described (Jiang et al. 1992). MCF-7 WS8 cells were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum, 1% glutaMAX, 1% AB/AM, 1% nonessential amino acids, and insulin (6 ng/mL). Cells utilized in assays were changed to an experimental media made as above except with phenol-red free media with 10% stripped FBS and 0.5% AB/AM 72 hours before plating.

2.3 **Analysis of Estrogen Metabolism by LC-MS/MS:**

2.3.1 **No Estradiol during Treatment (Chapter 3):**

The methoxyestrone metabolites, 2-MeOE_1 and 4-MeOE_1, were measured by LC-MS/MS as indicators of the level of estrogen 2-hydroxylation and the 4-hydroxylation as previously described with modifications (Wang et al. 2016a, Dunlap et al. 2015a). Metabolite standards were obtained from Steraloids Inc. (Newport, RI). The internal standard, 4-MeOE_1-1,4,16,16-d4 was obtained from CDN Isotope (Pointe-Claire, Quebec). In previous studies, the LC-MS/MS method co-treated compounds/extracts with E_2 for 48 h (Wang et al. 2016a, Dunlap et al. 2015a). When applying this method,

RCE treatment yielded several LC-MS peaks that interfered with the 2-$MeOE_1$ and 4-$MeOE_1$ peaks. To eliminate this issue, a PBS washing step was added after treatment with RCE/isoflavones (48 h) and before incubation with E_2 alone (1 µM for 24 h.). In this modified method, cells were estrogen starved for 72 h and plated in 6-well plates in phenol-red free media at 3.5 x 10^5 cells/well (MCF-7) or 1.6 x 10^5 cells/well (MCF-10A). After 24 h, cells were incubated for 48 h with RCE/isoflavones. Cells were washed with PBS then incubated with 1 µM E_2 for 24 h. Cell media were collected and spiked with 0.4 nM internal standard (4-$MeOE_1$-d4) and 2 mM ascorbic acid. The media were then extracted with dichloromethane (2 x 4 mL). The combined organic layers were then dried under nitrogen. Dansylation was performed as described previously (Wang et al. 2016a, Dunlap et al. 2015a) with $NaHCO_3$ buffer (75 µL, 0.1 M, pH 9.5) and 75 µL of dansyl chloride in acetone (1.25 mg/mL). Derivatized samples were analyzed by positive ion electrospray tandem mass spectrometry using an Agilent 1200 series nano flow LC system (Agilent Technologies, Santa Clara, CA) coupled to an AB SCIEX QTRAPTM 5500 System (AB SCIEX, Framingham, MA) as described previously (Tsuchiya et al. 2004b, Angus, Larsen, and Jefcoate 1999). Quantitation was performed using Analyst software (Applied Biosystems, Forster City, CA) and the data were normalized to the DMSO treatment.

2.3.2 **Pre/cotreatment with E_2 during treatment (Chapter 6):**

To match the conditions of E_2 containing models as much as possible, MCF-7/WS8 cells were plated in a monolayer as above with E_2 (1 µM) and incubated for 72 h.

After 72 h, cells were treated with fresh E_2 and extract or compound. After 24 h, media were collected, and analysis was performed as described above.

2.4 Mammosphere formation:

MCF-7 WS8 cells were plated at a density of 10,000 cells/well at a volume of 100 µL/well in a low adhesion round bottom plate (Corning), centrifuged at 1000 RPM for 5 minutes and set in the humid incubator at 5% CO2 at 37 C and for 72 h. Cells were treated to a total volume of 200 µL/well and placed in the incubator for 24 hours before reading.

2.5 Determination of spheroid/mammosphere diameters

Mammospheres were grown and treated as specified above. Before any other analysis, the 96 well low adhesion plate containing the spheroids and media was placed in the GelCount reader (Oxford Optronix, Oxford, UK). Pictures were taken of each well and diameters were determined via GelCount software (version 1.4).

2.6 Analysis of Gene Expression by RT-qPCR:

2.6.1 Mammosphere:

Ambion Cells to C T TM 1-Step TaqMan TM kit from Thermo-Fisher Scientific was used for qRT-PCR (real time-reverse transcription polymerase chain reaction) according to manufacturer's protocol. 10,000 cells were plated in concave and clear bottomed 96-well plate, centrifuged at 1000 rpm for 5 minutes and incubated for 3 days. After 72 hours cells were treated, and PCR was performed similarly to the 2D-qRT-PCR. An additional plate for MTT was grown and treated as with the PCR plate, with MTT solution added and incubated for 2 hours before reading absorbance and

normalizing between PCR preformed in cell in a monolayer (2D) and those in a mammosphere (3D).

2.6.2 Monolayer**:**

Ambion Cells to C T TM 1-Step TaqMan TM kit from Thermo-Fisher Scientific was used for qRT-PCR (real time-reverse transcription polymerase chain reaction) according to manufacturer's protocol. MCF-7:WS8 cells were plated in clear 96-well plates at a density of about 40,000 cells per well and incubated at 37 °C in 5% CO_2 for 2 days. After 48 hours, cells were treated with compounds alone or in combination with E_2 and incubated for another 24 hours. After 24 hours cells were washed and lysed with a total of 45 µL solution for 5 minutes before adding 5 µL of stop solution for 5 minutes then freezing overnight. The next day, 2 µL of thawed lysate for each treatment was mixed with 5 µL of TaqMan 1-Step RT-PCRmaster mix, 1 µL of HPRT1 (Hs02800695_m1) primer with a VIC-MGB probe, 1 µL of either *CYP1A1* (Hs00153120_m1) or *CYP1B1* (Hs00164383_m1) primers with FAM-MGB probes, and 11 µL of PCR-grade water. Solutions of mRNA were read on a StepOnePlus TM fluorescence detection system (Thermo Fisher, Waltham, MA, USA) to produce cDNA CT fold change values for each treatment. Data for three trials of triplicates were analyzed according to the comparative C T method (ΔΔC T) and expressed as fold induction relative to DMSO treated cells.

2.7 XRE-Luciferase Reporter Assay (Monolayer):

HC-04 and/or MCF-7 cells were plated in 12-well plates overnight and transfected at 70% confluency with luciferase and renilla plasmids (Promega, Madison, WI), XRE pGL4.43, and pRL-TK vector, respectively, using Lipofectamine 2000 reagent for 6 h.

Cells were treated with the extract/isoflavones for 24 h and lysed with buffer. The lysates were analyzed for luciferase activity according to Promega's Dual-Luciferase reporter assay system protocol using BioTek (Winooski, VT) SYNERGY TM multi-mode reader system (Dowsett et al. 2015). For experiments with ICI 182,780 in MCF-7 cells, the cells were pretreated with ICI 182,780 for 2 h and incubated with compounds for an additional 24 h before the cells were lysed and analyzed for luciferase activity.

2.8 Induction of estrogen-responsive alkaline phosphatase (AP) in Ishikawa cells (Monolayer):

Ishikawa cells (5×10^4 cells/well) were pre-incubated in 96 well plates in estrogen-free medium for 24 h. Tested samples were dissolved in DMSO. These were added at different concentrations. To determine the anti-estrogenic activity, treatments were performed in the presence of E_2 (2 nM), well above its EC_{50}. Plates were incubated at 37°C for 96 h. Cells were washed with PBS and lysed by adding 50 µL of 0.01% Triton X-100 in 0.1 M Tris buffer (pH 9.8) followed by a cycle of freeze and thaw at −80°C and 37°C, respectively. *p*-Nitrophenol phosphate (phosphatase substrate) (18 µM) was added to each well and the alkaline phosphatase activity was measured by reading the formation of *p*-nitrophenol at 405 nm every 15 s with a 10 s shake between readings for 16 readings using a Power Wave 200 microplate scanning spectrophotometer (Bio-Tek Instruments, Winooski, VT). The maximum slope of the kinetic curve for every experiment well was calculated. The fold induction of alkaline phosphatase for every treatment, compared to that of the E_2 control was represented as estrogenic activity and calculated as described previously (Pisha and Pezzuto 1997). Anti-estrogenic activity

was stated as the fold induction of alkaline phosphatase compared to background induction control (Pisha and Pezzuto 1997).

2.9 7-ethoxy-resorufin-O-deethylase activity assay

2.9.1 Mammosphere:

Mammospheres were formed as stated above (2.4) and treated for 24 hours with (A) treatment alone or (B) treatment +TMS (1 μM) in triplicate. After 24 h treatment, spheroid diameter was measured in gel cell counter. Cells were washed with PBS (100 μL/well). In the dark, (A) 5 μM 7-ethoxyresorufin and 3 μM salicylamide alone or (B) + TMS (1 μM) solutions in PBS were added and the fluorescence was measured every minute with excitation at 530 nm and emission at 590 nm for 45 min at 37 °C with a BioTek (Winooski, VT) SYNERGY TM multi-mode reader system. Fluorescence/time reading was linear for more than 30 min between 15 min and 45 min. The reaction rate was determined from the slope of the linear regression curves plotted with data points measured between 15 min and 45 min. The reaction rate was normalized to sphere diameter and then to negative control (DMSO; 0.1%) and reported as fold change except in where otherwise specified.

2.9.2 Monolayer:

EROD assay measuring P450 1 enzyme activity was conducted both in cells as previously described (Wang et al. 2016a, Dunlap et al. 2015a). Briefly, 1×10^5 cells/well were plated and treated with extract or compounds for 24 h, cells were washed with PBS and incubated with 2.5 μM 7-ethoxyresorufin and 1.5 μM salicylamide in PBS at 37 °C. Fluorescence was measured as above (2.8.1). Fluorescence was measured every

minute after 5 s of mixing for 25 min at 37 °C. The reaction rate was determined from the slope of the linear regression curves plotted with data points measured between 5 min and 20 min. The reaction rate was normalized to negative control (DMSO; 0.1%) and reported as fold change except in Figure 10B in which only the reaction rate (Fluorescence Units/min) is shown.

2.10 In silico docking analysis:

2.10.1 Estrogen Receptor α (ERα)

The binding site of ERα was obtained from Protein Data Bank (PDB ID: 1x7r) and uploaded to Molecular Operating Environment (MOE; Chemical Computing Group, version 2016.0208). All unnecessary waters were removed, and structure was prepared using the quick prep option in MOE. Compounds were docked with triangle matcher placement with London dG scoring and induced fit refinement with GBVI/WSA dG scoring. Figures with surfaces are shown with the docked compound in molecular surface showing hydrophobicity and lipophilicity of the binding site.

2.10.2 Aryl Hydrocarbon Receptor (AhR)

At the time, when this model was validated, there was no crystal structures of the binding site of human AhR available in the Protein Data Bank. Therefore, a homology was obtained from SWISS MODEL database (ID: P35869) and uploaded to Molecular Operating Environment (MOE; Chemical Computing Group, version 2016.0208). The structure was prepared using the quick prep option in MOE. The positive control, TCDD, was docked to potential binding sites. The site in which strong AhR agonists 2,3,7,8-Tetrachlorodibenzodioxin (TCDD) bound with the best binding score was confirmed with

a literature search and defined as the binding site. Compounds were docked into this binding site with triangle matcher placement with London dG scoring and induced fit refinement with GBVI/WSA dG scoring. Figures with surfaces are shown with the docked compound in molecular surface showing hydrophobicity and lipophilicity of the binding site.

2.11 Statistical Analysis.

Unless otherwise specified, the analyzed data were normalized to DMSO (0.1%). All replicates (not means of each run) were combined and confirmed to have normal distribution with D'Agostino-Pearson test. Significance was determined using unpaired t-test, comparing test sample data to the DMSO control sample unless otherwise stated. $p< 0.05$ indicates significance (*) Data are expressed as mean ± SEM of at least three independent experiments. *$p< 0.05$ indicates significance (*). IC_{50}/EC_{50}'s were determined as means and standard deviations of IC_{50}s of three separate runs.

3 RED CLOVER ARYL HYDROCARBON RECEPTOR (AHR) AND ESTROGEN RECEPTOR (ER) AGONISTS ENHANCE GENOTOXIC ESTROGEN METABOLISM

(Reprinted in part with formatting changes from: Dunlap, Tareisha L., Caitlin E. Howell, Nita Mukand, Shao-Nong Chen, Guido F. Pauli, Birgit M. Dietz, and Judy L. Bolton. (2017). "Red Clover Aryl Hydrocarbon Receptor (AhR) and Estrogen Receptor (ER) Agonists Enhance Genotoxic Estrogen Metabolism." Chemical Research in Toxicology 30 (11):2084-2092. doi: 10.1021/acs.chemrestox.7b00237. Paper can be accessed at https://pubs.acs.org/doi/10.1021/acs.chemrestox.7b00237. Further permissions related to the material excerpted should be directed to the American Chemical Society).

3.1 Rationale and hypothesis

Breast cancer remains the most prevalent cancer among women, with an estimated quarter of a million breast cancer diagnoses in 2015 alone (Siegel, Miller, and Jemal 2016b). Estrogens can initiate cancer when their binding to the ERα leads to increased cell proliferation and the likelihood of DNA mutations (hormonal pathway of carcinogenesis) (Wang et al. 2016a). Breast cancer risk is also influenced by estrogen metabolism, and the genotoxic estrogen quinones formed in this process (Sampson, Falk, Schairer, Moore, Fuhrman, Dallal, Bauer, Dorgan, Shu, and Zheng 2017, Bolton and Dunlap 2017b, Yager 2015) can be modulated by dietary means, including botanical dietary supplements (BDSs) (Wang et al. 2016a, Dunlap et al. 2015a, Reding et al. 2014). As HT is associated with an increased risk of breast cancer, many women use BDSs, which are perceived as safer alternatives for the relief of menopausal

symptoms (Wang et al. 2016a, Van Breemen 2015, Manson et al. 2013, Bolton 2016). However, efficacy claims are not only disallowed for BDSs, but also remain questionable. In particular, the effect of estrogenic BDSs on estrogen metabolism is unknown.

Ligand activation of the aryl hydrocarbon receptor (AhR) followed by cooperative binding of AhR and the aryl hydrocarbon receptor nuclear translocator (ARNT) to xenobiotic response elements (XREs) upregulates P450 1B1 (Shimada et al. 1998), which metabolizes estrogens to 4-hydroxylated catechols (4-$OHE_{1/2}$). These catechols are oxidized to genotoxic, unstable quinones that form depurinating DNA adducts and apurinic sites, collectively resulting in carcinogenesis via the estrogen chemical carcinogenesis pathway (Figure 2) (Bolton and Dunlap 2017b, Cavalieri and Rogan 2016a). Given that 4-OHE_2 can transform estrogen receptor-negative (ER-) cells into a malignant phenotype (Park et al. 2016, Park, Na, and Surh 2012, Hemachandra et al. 2012), activation of the chemical pathway is likely an important event in breast cancer initiation and/or promotion. AhR signaling also activates P450 1A1, which metabolizes estrogens to 2-hydroxylated metabolites (2-$OHE_{1/2}$, Figure 2) (Yager and Davidson 2006a, Bolton and Thatcher 2007, Cavalieri and Rogan 2016b). However, the 2-hydroxylation pathway is negatively correlated with breast cancer risk and can be regarded as a detoxification pathway (Sampson, Falk, Schairer, Moore, Fuhrman, Dallal, Bauer, Dorgan, Shu, and Zheng 2017). Additionally, the 2-hydroxylated catechol estrogens reduce E_2-induced cell proliferation (Gupta, McDougal, and Safe 1998). Catechol-*O*-methyltransferase (COMT) metabolizes 2-$OHE_{1/2}$ to 2-methoxyestrone (2-

$MeOE_{1/2}$), and 2-$MeOE_{1/2}$ inhibits E_2-induced proliferation of breast cancer cells (Mueck and Seeger 2010). The lack of information on the modulation of estrogen metabolism by botanicals used for menopausal symptom relief motivated the present study.

Several popular BDSs used for menopausal symptom management contain constituents that alter P450 1A1 and P450 1B1 gene expression (i.e., *CYP1A1* and *CYP1B1*) or inhibit these enzymes in various cell lines (Dong et al. 2016, Snelten, Dietz, and Bolton 2012). However, studies investigating the broader effect of these BDSs on estrogen metabolism are scarce. Previous studies reported that two popular and relevant BDSs, licorice and hops, can modulate estrogen metabolism in breast cells. Evidence was built on the LC-MS/MS detection of the methoxy estrogen metabolites, 2-$MeOE_1$ (non-toxic biomarker) and 4-$MeOE_1$ (genotoxic biomarker, Figure 2) (Wang et al. 2016a, Dunlap et al. 2015a). These studies found that an extract of one pharmacopoeial licorice species, Glycyrrhiza inflata, and its marker compound, licochalcone A (AhR antagonist), decreased 4-$MeOE_1$ and *CYP1B1* expression in MCF-10A cells (Dunlap et al. 2015a). Furthermore, in both MCF-10A cells and MCF-7 cells, hops and its bioactive constituent, 6-prenylnaringenin (AhR agonist), preferentially induced the 2-hydroxylation detoxification pathway (2-$MeOE_1$ > 4-$MeOE_1$) (Wang et al. 2016a). Phytoestrogens, such as GN, DZ, liquiritigenin, and S-equol, reportedly modulated *CYP1A1* and *CYP1B1* expression through both ERα and AhR mechanisms in ER+ MCF-7 cells (Gong et al. 2016). Hence, in ER+ breast tissue, botanical constituents can target both ERα and AhR and significantly alter *CYP1A1* and/or *CYP1B1* expression (Figure 2). One key insight from these previous studies is that the

impact of BDSs used for menopausal women's health on estrogen metabolism in breast cells should be studied systematically in different models, including ER+ and ER- cells, as outcomes vary according to cell/tissue type.

The current study used both non-tumorigenic ER- breast epithelial cells (MCF-10A) and ER+ breast cancer cells (MCF-7) as a model for women with ER+ breast cancer cells to determine the effects of a red clover extract (RCE) and four of its bioactive marker isoflavones, GN, DZ, BA, and FN, on estrogen metabolism. The dried flowering above ground parts of red clover (*Trifolium pratense L., Fabaceae*) have been used traditionally as an expectorant and against skin inflammation (Kolodziejczyk-Czepas 2012); however, because of its estrogenic isoflavone content, the predominant, current use of the extract is for menopausal symptoms (Geller et al. 2009, Franco et al. 2016). GN and DZ are estrogenic isoflavones from red clover and soy that preferentially activate ERβ (Overk, Yao, Chadwick, Nikolic, Sun, Cuendet, Deng, Hedayat, Pauli, Farnsworth, et al. 2005). BA and FN are the 4'-methoxy ether analogs of GN and DZ that undergo P450 catalyzed O-demethylation to GN and DZ *in vivo* (Peterson et al. 1996, Choi et al. 2008). In addition, BA and FN are AhR agonists, which can induce P450 metabolism (Han, Jeong, and Jeong 2007, Han, Kim, and Jeong 2006a, Medjakovic and Jungbauer 2008, Chan, Wang, and Leung 2003); however, to date no studies have focused on their effect on estrogen metabolism in breast cells. The current study fills this gap by evaluating the effect of chemically standardized RCE and marker isoflavones on estrogen metabolites and *CYP1A1*/*CYP1B1* expression in both MCF-

10A and MCF-7 cells. The goal of this study was to better understand the impact of red clover dietary supplements on the alteration of estrogen metabolism.

3.2 Results

3.2.1 RCE and Its Major Isoflavones Do Not Increase Estrogen Metabolism in MCF-10A Cells; RCE Downregulates *CYP1A1* and Increases Genotoxic Estrogen Metabolism in MCF-7 Cells.

In order to study the effect of RCE on estrogen metabolism in breast tissue, ER- breast epithelial cells (MCF-10A) were used a ER- breast cell model. The current study used an LC-MS/MS method to measure methoxyestrone metabolites that was slightly modified (Chapter 2) (Wang et al. 2016a) to determine the effect of RCE and its isoflavones on estrogen metabolism. RCE (Figure 4A) and its major isoflavones, GN, DZ, BA, and, FN, showed no effect in this model. These data suggested that in ER- cells, RCE has no effect on the chemical estrogen carcinogenesis pathway. To determine whether RCE and/or its isoflavones modulate estrogen metabolism in an ER+ breast cancer model, the same experiments performed in MCF-10A cells were carried out in MCF-7 cells. Interestingly, here RCE (10 µg/mL) caused an increase in both estrogen 2-hydroxylation and 4-hydroxylation (Figure 4A). Levels of 4-$MeOE_1$ reached 10-fold induction and represented nearly double of the 2-$MeOE_1$ levels observed after two-days treatment with RCE (10 µg/mL). Similarly, *CYP1B1* expression (five-fold) was twice that of *CYP1A1* expression after 24 h RCE treatment (10 µg/mL; Figure 4B). Interestingly, at lower concentrations (< 5 µg/mL), RCE significantly downregulated *CYP1A1* expression below basal levels (Figure 4B). As a result, E_2 was predominately

metabolized by P450 1B1 to potentially genotoxic catechols in MCF-7 cells under these conditions.

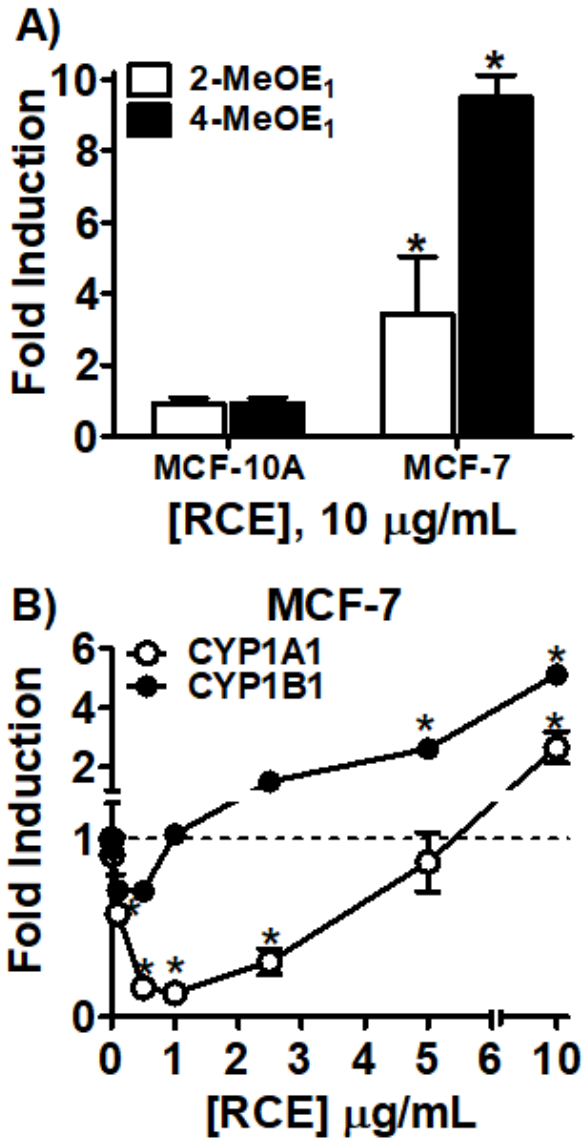

Figure 4: LC-MS/MS analysis of methoxyestrone metabolites (2-MeOE$_1$, 4-MeOE$_1$) in MCF-10A and MCF-7 cells and *CYP1A1*/*CYP1B1* levels in MCF-7 cells.
A) MCF-10A and MCF-7 cells were treated with RCE (10 µg/mL) and for 48 h and then 24 h with E_2 (1 µM). B) *CYP1A1* and *CYP1B1* expression levels were determined after 24 h treatment with RCE in MCF-7 cells by qPCR analysis. Normalized to and t-test compared to DMSO (0.1%) negative control. *p<0.05.

3.2.2 Isoflavones Preferentially Increase 4-MeOE_1 Metabolites.

To determine which isoflavones might be responsible for RCE's stimulating effect on estrogen metabolism, MCF-7 cells were treated with different concentrations of GN, DZ, BA, and FN. GN and DZ had no significant effect on the 2-hydroxylation pathway (Figure 5A). Overall, GN and DZ at concentrations ranging from 1 µM to 10 µM significantly increased the production of 4-MeOE_1 by at least five-fold (Figure 5B). In contrast, BA and FN (10 µM), increased 4-MeOE_1 more than 2-MeOE_1 metabolites, at least eight and five times, respectively (Figure 5C and D). The maximum induction of 4-MeOE_1 by BA and FN (10 µM) was 20-fold and 30-fold, respectively.

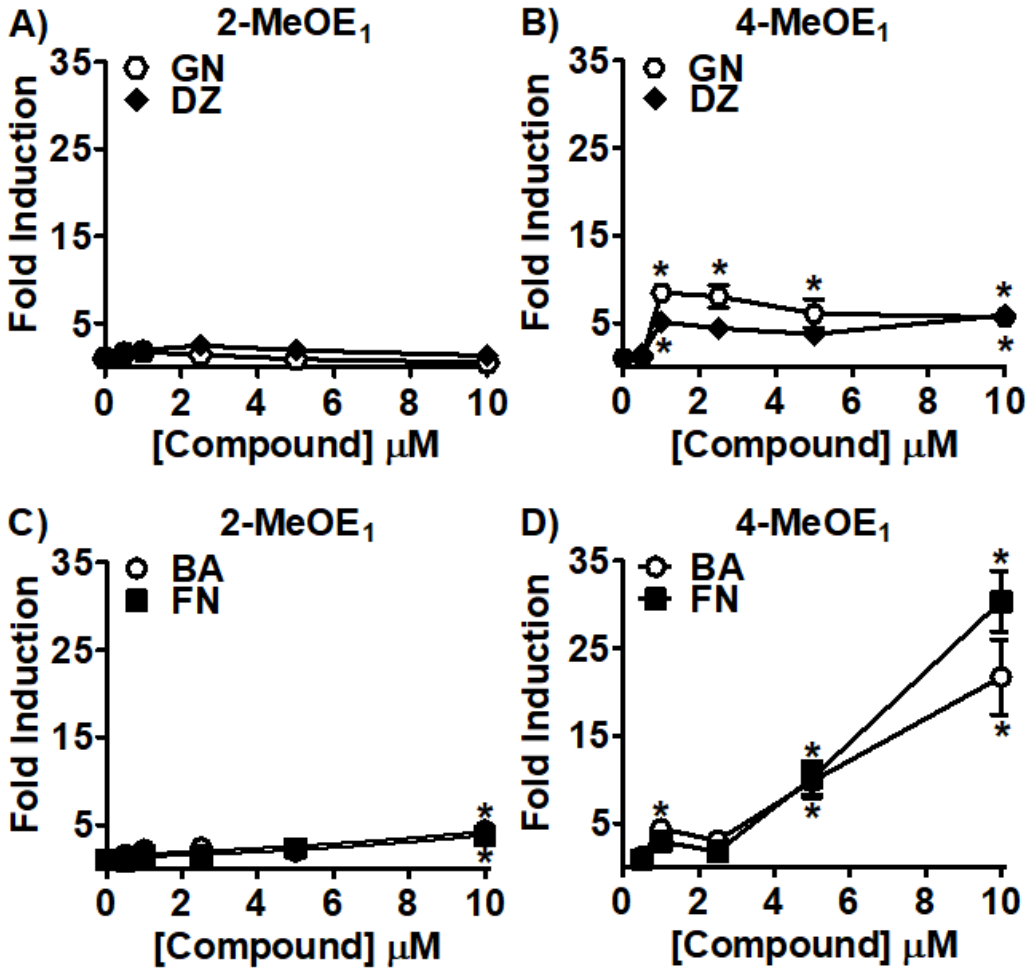

Figure 5: LC-MS/MS analysis of methoxyestrone metabolites (2-MeOE$_1$, 4-MeOE$_1$) in MCF-7 cells after treatment with isoflavones.
MCF-7 cells were treated for 48 h with the isoflavones, GN, DZ, BA, and FN, followed by 24 h treatment with E_2 (1 μM). 2-MeOE$_1$ and 4-MeOE$_1$ metabolites were analyzed by LC-MS/MS. A) and B) Effect of the ER agonists, GN and DZ, on A) 2-MeOE$_1$ and B) 4-MeOE$_1$ metabolites. C) and D) Effect of the AhR agonists, BA and FN, on C) 2-MeOE$_1$ and D) 4-MeOE$_1$ metabolites. Normalized to and t-test compared to DMSO (0.1%) negative control. *$p<0.05$.

3.2.3 **Isoflavones Preferentially Increase *CYP1B1* Expression; ER Agonists Significantly Downregulate *CYP1A1*.**

As Trifolium isoflavones increase the P450 1B1 catalyzed genotoxic pathway and have little to no effect on the P450 1A1 catalyzed detoxification pathway, their effect on *CYP1A1* and *CYP1B1* gene expression was measured to study these differential effects in more depth. GN and DZ dose-dependently downregulated *CYP1A1* expression (Figure 6A). GN and DZ caused a small increase in *CYP1B1* expression to two-fold at the highest test concentration (20 µM, Figure 6B). In contrast, BA and FN upregulated *CYP1A1* expression to eight- and four-fold, respectively, at 20 µM (Figure 6C). Even larger induction was observed with *CYP1B1* expression (Figure 6D).

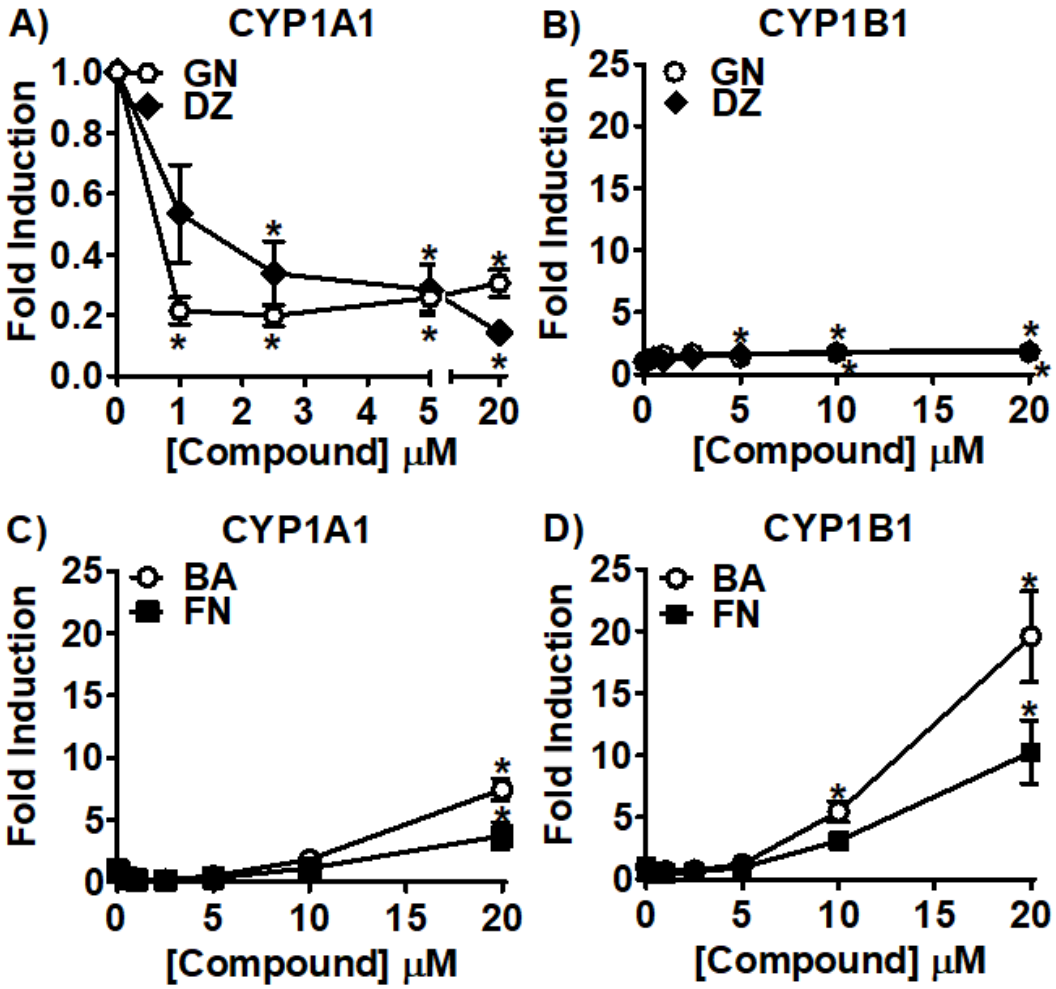

Figure 6: *CYP1A1*/*CYP1B1* expression analyzed by qPCR in MCF-7 cells after treatment with isoflavones.
MCF-7 cells were treated for 24 h with the isoflavones, GN, DZ, BA, and FN and *CYP1A1* and *CYP1B1* expression was analyzed by qPCR. A) and B) Effect of the ER agonists, GN and DZ, on A) *CYP1A1* and B) *CYP1B1* expression and C) and D) effect of the AhR agonists, BA and FN, on C) *CYP1A1* and D) *CYP1B1* expression. Normalized to and T-test compared to DMSO (0.1%) negative control. *p<0.05.

3.2.4 **The Estrogen Receptor Antagonist ICI 182,780 Abolishes GN and DZ-Induced Downregulation of *CYP1A1* but Increases BA and FN Mediated *CYP1A1* Upregulation.**

The role of ERα on *CYP1A1* and *CYP1B1* expression was investigated by treating MCF-7 cells with ER agonists (E_2, GN, and DZ), AhR agonists (TCDD, BA, and FN), and an ER agonist with an AhR agonist (TCDD, E_2) with and without ICI 182,780 (Figure 7). E_2 (1 nM) alone significantly downregulated *CYP1A1* expression (Figure 7A) and had no effect on *CYP1B1* (Figure 5B). As expected, TCDD increased *CYP1A1* expression (425-fold, Figure 7C) and *CYP1B1* expression (28-fold, Figure 7D). E_2 decreased TCDD-induced *CYP1A1* expression from 425-fold to 220-fold (Figure 7C) and had no effect on TCDD-induced *CYP1B1* expression (Figure 7D). ICI 182,780 treatment alone modestly increased *CYP1A1* (Figure 7A) and *CYP1B1* expression (Figure 7B). Similar to E_2, pretreatment with ICI 182,780 followed by GN and DZ, effectively eliminated *CYP1A1* downregulation by these estrogenic isoflavones (Figure 5A). After pretreatment, ICI 182,780 was even more effective at potentiating BA and FN-mediated *CYP1A1* upregulation, increasing it from seven- and six-fold, respectively, to 55- and 40-fold (Figure 7C). In comparison with isoflavones alone, ICI 182,780 pretreatment did not significantly modulate *CYP1B1* expression (Figure 7B, Figure 7D). When ICI was added to TCDD co-treated with E_2 and TCDD treatment alone, both *CYP1A1* (Figure 7C) and *CYP1B1* fold induction (Figure 7D) greatly increased. These data suggest that ERα specifically influences the effect of RCE and its isoflavones on *CYP1A1* expression, without altering *CYP1B1*.

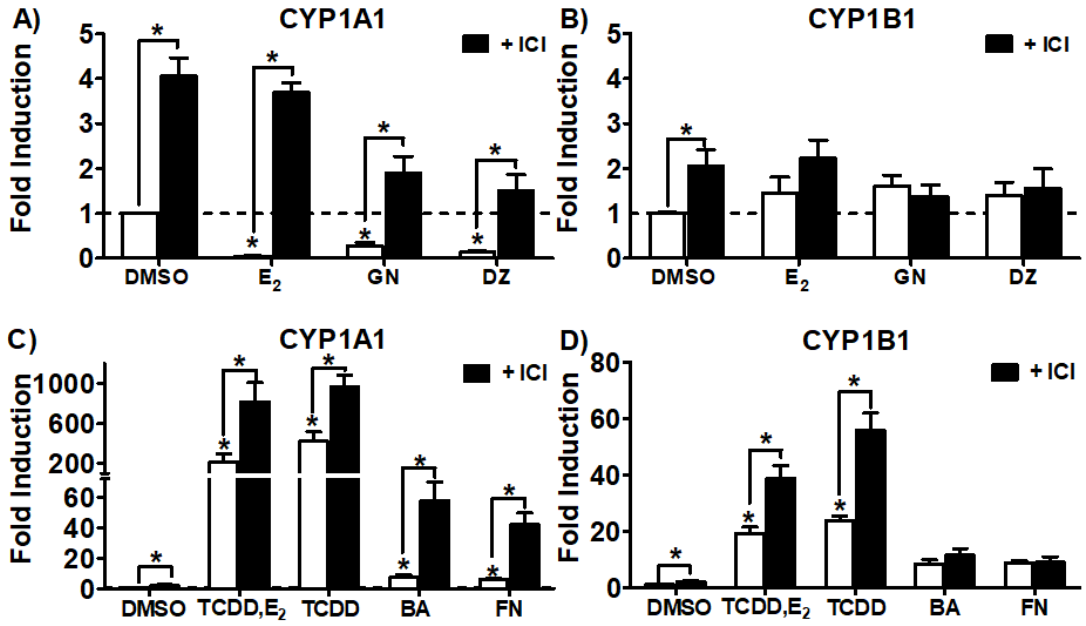

Figure 7: *CYP1A1*/*CYP1B1* expression analyzed by qPCR in MCF-7 cells after treatment with isoflavones.
A) Cells were pretreated with ICI (1 µM) for 2 h and ER agonists [E_2 (1 nM), and GN and DZ (10 µM)] were added for an additional 24 h before qPCR analysis of *CYP1A1* and B) *CYP1B1* levels. Treatments with AhR agonists [TCDD (10 nM), TCDD (10 nM) + E_2 (1 nM), BA (10 µM) and FN (10 µM)] were added for an additional 24 h after 2 h ICI 182,780 pretreatment before qPCR analysis of C) *CYP1A1* and D) *CYP1B1* levels. Normalized to and t-test compared cells treated with both compound and ICI to that treated with compound alone. *p<0.05.

3.2.5 **The AhR Agonists, BA and FN, Significantly Induced XRE-Luciferase Activity, Which Can Be Further Induced by ICI 182,780.**

To further elucidate mechanistic differences between the tested isoflavones and to compare AhR transcriptional activation by isoflavones, XRE-luciferase reporter activity was analyzed (Figure 8A). HC-04 cells were transfected with an XRE-luciferase

reporter plasmid and treated with isoflavones for 24 h. Both GN and DZ had no significant effect, while BA and FN significantly increased activity to five- and seven-fold. XRE-luciferase activity was also measured in MCF-7 cells; however, only FN had a significant effect (Figure 8B). Because ERα can suppress AhR activation (Gong et al. 2016), the MCF-7 cells were pretreated with ICI 182,780 before isoflavone treatments and XRE-luciferase activity was determined. Interestingly, the XRE-luciferase fold induction by isoflavone treatment more than doubled with ICI 182,780 pretreatment (Figure 8B). ICI 182,780 alone had no effect on XRE-luciferase reporter activity. These data confirmed that BA and FN act as AhR agonists and that ERα downregulates XRE-reporter activity.

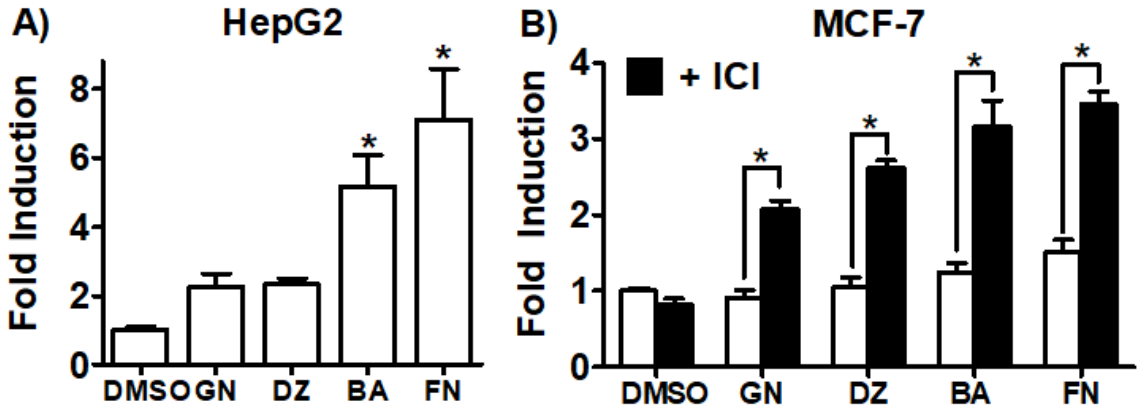

Figure 8: XRE-luciferase reporter activity in HC-04 and MCF-7 cells after treatment with isoflavones.
A) XRE-luciferase reporter activity was analyzed after 24 h treatment with isoflavones (10 µM), BA, FN, GN, and DZ, in A) HC-04 cells and B) in MCF-7 cells. ICI 182,780 (1 µM) was added 2 h before treatment with compounds for an additional 24 h before analysis of XRE-luciferase reporter activity. Normalized to and T-test compared to DMSO negative control. *$p<0.05$.

3.3 Discussion

Although both P450 1A1 and P450 1B1 genes, *CYP1A1* and *CYP1B1*, respectively, are coordinately upregulated by AhR agonists, ER agonists differentially regulate these genes (Huang, Chiang, and Chen 2011) 37. As many botanicals used for menopausal symptoms, like red clover, contain AhR agonists that can increase estrogen metabolism along with phytoestrogens (Spink, Spink, Cao, DePasquale, Pentecost, Fasco, Li, and Sutter 1998) 38 that may downregulate *CYP1A1* similarly to E_2 (Shull et al. 1997) 37, evaluating their effect on estrogen metabolism *in vitro* is a means of enhancing the prediction of safety *in vivo* . Because the effect of RCE and its isoflavones on estrogen metabolism had not been previously investigated, we studied their effects in two different models. MCF-10A cells were used as an ER- model while MCF-7 cells were an ERα+ model. The ER expression levels in MCF-10A are much lower than in ER+ breast cancer cell lines and are thus considered ER-{Spink, 1998 #27}.

The study outcome showed that RCE did not modulate estrogen metabolism in ER- MCF-10A cells (Figure 4A). Red clover BDSs are, therefore, most likely safe, a conclusion also supported by several *in vivo* studies analyzing red clover's hormonal effects (Geller et al. 2009, Burdette et al. 2002, Powles et al. 2008, Atkinson et al. 2004). An RCE (750 mg/kg/day) standardized to 15% isoflavones and given by gavage to ovariectomized rats did not increase proliferation in the breast (Burdette et al. 2002). Available in the form of a commercial dietary supplement (Promensil), a red clover preparation was administered for 3 years to healthy women with a family history of

breast cancer. In line with other evidence, Promensil did not increase breast density, which is known to increase the risk of breast cancer (Powles et al. 2008). Another study by Atkinson et al. found no difference in breast density or E_2 levels (Atkinson et al. 2004). Furthermore, it appears that isoflavones may even decrease breast cancer risk (Boucher et al. 2013). The association between breast cancer risk and the use of isoflavone supplements has also been analyzed among postmenopausal women. Using high content isoflavone supplements, at least 3 of the 28 total isoflavone dietary supplements included in the study, or any isoflavone supplement consumed for over 5 years were associated with a decreased risk in breast cancer (Boucher et al. 2013). Red clover isoflavones possess other beneficial effects that may contribute to a positive safety profile in healthy women. The ERβ selectivity of GN and DZ may provide a better safety profile than classical estrogens (Dietz et al. 2016, Amer et al. 2010). Additionally, GN inhibited DMBA-induced DNA oxidative damage and strand breaks in MCF-10A cells (Leung et al. 2008).

However, other studies reported potential harmful effects of red clover phytoestrogens, such as stimulation of breast cancer cell proliferation and tumor growth at concentrations as low as 1 nM (de Lemos 2001). Hsieh et al. showed that GN caused cell growth in MCF-7 cells, increased mammary gland growth in ovariectomized mice, and increased MCF-7 xenograft tumors (Hsieh et al. 1998). Downregulation of COMT mRNA and activity in MCF-7 cells has also been reported for phytoestrogens GN and DZ, which led to decrease in methylation of the genotoxic 4-OHE_2 metabolite (Lehmann, Jiang, and Wagner 2008). In this study, MCF-7 breast cancer cells were also

used to investigate red clover's safety, and the results were compared to those from MCF-10A cells. It was noted that AhR responsiveness was much higher in MCF-7 cells than in MCF-10A cells. This has been observed when TCDD increased 2-$MeOE_1$ and 4-$MeOE_1$ levels in MCF-7 cells at least 4- and 7-fold more than in MCF-10A cells (Spink, Spink, Cao, DePasquale, Pentecost, Fasco, Li, and Sutter 1998). Subsequently, this study found that, while RCE caused an increase in overall estrogen oxidative metabolism, the P450 1B1-mediated genotoxic pathway was increased more than the P450 1A1 detoxification pathway in MCF-7 cells (Figure 4). Thus, RCE is predicted to be safe in ER- breast tissue, yet it may modulate estrogen metabolism to increase genotoxic metabolites in ER+ breast tissue.

In MCF-7 cells, the individual effects of red clover isoflavones on estrogen metabolism in this study were determined as well. We previously determined that natural AhR agonists, such as 6-prenylnaringenin, increase estrogen metabolism through AhR activation (Wang et al. 2016a, Dunlap et al. 2015a). In the literature, BA and FN are reported as being AhR agonists (Han, Jeong, and Jeong 2007, Han, Kim, and Jeong 2006a), but whether GN and DZ activate AhR is controversial. Comparing relative AhR activation potencies, Bialesova et al. reported AhR activation by BA, FN, and GN (100 μM) to be 309-, 108-, and 27-fold, respectively, with DZ having no effect (Bialesova et al. 2015). A number of flavonoids and plant-derived indoles were tested in an assay to measure transcriptional activation of the AhR/ARNT dimer using an XRE reporter plasmid in yeast (Zhang, Qin, and Safe 2003). The EC_{50}s of BA and FN were 130 and 250 nM, respectively, designating them as being among the most potent

compounds tested (Zhang, Qin, and Safe 2003). EC_{50}s were not reported for GN and DZ because the compounds were not potent enough for AhR transcriptional activation to be detected. However, GN and DZ (10 μM) significantly activated AhR in Hepa-1 cells to 12- and 14-fold, yet their induction of AhR activation did not reach 2-fold in MCF-7 cells (Zhang, Qin, and Safe 2003). In the current study, GN and DZ (10 μM) increased XRE-luciferase reporter activity in HC-04 cells to 2-fold; however, the induction was 4 times lower than after BA and FN treatments (Figure 8A). AhR activation leading to *CYP1A1* and *CYP1B1* induction after isoflavone treatments has been reported (Gong et al. 2016, Han, Jeong, and Jeong 2007, Han, Kim, and Jeong 2006a), yet no studies have conducted a direct comparison of *CYP1A1* and *CYP1B1* induction by the four key marker isoflavones in MCF-7 cells. BA and FN (10 μM) reportedly increased *CYP1A1* expression in MCF-7 cells after 6 h (Han, Jeong, and Jeong 2007, Han, Kim, and Jeong 2006a) BA (10 μM) doubled *CYP1A1* expression and increased P450 1 specific activity 2.5-fold; however, *CYP1B1* expression was not determined in this study. Studies aimed at determining the effect of GN and DZ on *CYP1A1* and/or *CYP1B1* are conflicting. Gong et al. produced results showing that GN and DZ (1 μM) increased both *CYP1A1* and *CYP1B1* expression to 5- and 3-fold, respectively, in MCF-7 cells after 4 h (Gong et al. 2016). However, Wagner et al. reported, after 24 h, a decrease in *CYP1A1* with GN (1 μM) and DZ (10 μM) in estrogen sensitive MCF-7 cells (MCF-7 BUS); *CYP1B1* was not measured in that study either (Wagner, Jiang, and Lehmann 2008). Overall, the present outcomes agree with other studies by showing that BA and FN were more potent than GN and DZ in activating

CYP1A1 and *CYP1B1*. Even at the highest concentration tested, GN and DZ did not increase 2-MeOE$_1$ metabolites (Figure 5A) or *CYP1A1* (Figure 6A), and they only modestly increased *CYP1B1* (2-fold, Figure 4B). As BA and FN significantly increased both P450 1A1 and P450 1B1 pathways, they are most likely the reason that RCE increased estrogen metabolism in MCF-7 cells.

On the other hand, GN and DZ, as ER agonists, are most likely the reason that RCE downregulated *CYP1A1* at low concentrations in MCF-7 cells, (Figure 4B) and demonstrated a relatively weak effect on estrogen 2-hydroxylation compared to 4-hydroxylation (Figure 4A). Estrogens limit AhR activation through various mechanisms, such as degradation of AhR or depletion of its available cofactors (Safe, Wormke, and Samudio 2000). Furthermore, when E_2 activates ERα to displace AhR/ARNT, methylation on XREs in the *CYP1A1* promoter region occurs (Marques, Laflamme, and Gaudreau 2013). This mechanism specifically downregulates *CYP1A1* without affecting *CYP1B1* expression in MCF-7 cells (Marques, Laflamme, and Gaudreau 2013). In the current study, E_2 (1 nM) decreased basal and TCDD-induced *CYP1A1* expression (Figure 7A, Figure 7C). Moreover, Spink et al. reported that significant downregulation of TCDD-induced *CYP1A1* expression/2-MeOE$_2$ formation simultaneously occurred with upregulation of TCDD-induced *CYP1B1*/4-MeOE$_2$ formation after E_2 treatments (100 pM to 10 nM) (Spink et al. 2003). Similarly, GN and DZ not only downregulated *CYP1A1* but also increased *CYP1B1*/4-MeOE$_1$ formation. However, it is likely but not certain that this increase in the 4-hydroxylation pathway by GN and DZ was mainly ERα mediated. GN and DZ are weak AhR agonists in MCF-7 cells (Zhang, Qin, and Safe 2003), and

studies regarding whether E_2 modulates *CYP1B1* expression are inconclusive (Gong et al. 2016, Marques, Laflamme, and Gaudreau 2013, Tsuchiya et al. 2004a, Beischlag and Perdew 2005). In the current study, E_2 (1 nM) slightly, yet not significantly, increased basal *CYP1B1* expression, but it produced no effect on TCDD-induced *CYP1B1* expression (Figure 7D). Some literature reports have shown that in the presence of E_2, TCDD-induced *CYP1B1* levels were not affected (Marques, Laflamme, and Gaudreau 2013, Beischlag and Perdew 2005). However, Gong et al. observed that basal *CYP1B1* levels were decreased (Gong et al. 2016), while others saw an increase in *CYP1B1* expression with E_2 treatment (Tsuchiya et al. 2004a, Beischlag and Perdew 2005). Increased *CYP1B1* expression by E_2 may occur through an estrogen response element (ERE) located in the promoter region of the *CYP1B1* gene (Tsuchiya et al. 2004a). Thus, upregulation of *CYP1B1* by GN and DZ could occur through ER and/or AhR mechanism(s) to increase the 4-hydroxylation of estrogens, yet downregulation of *CYP1A1* by GN and DZ through an ERα-mediated mechanism was most likely the reason that RCE reduced *CYP1A1* expression and thus only weakly induced 2-hydroxylation compared to 4-hydroxylation in MCF-7 cells.

It is probable that experimental conditions or cell type/context influenced the effect of estrogenic compounds on AhR activation. For instance, the concentration of E_2 in media, length of E_2 deprivation (Spink et al. 2003), and the well-documented differences in MCF-7 cell lines (Osborne, Hobbs, and Trent 1987, Nugoli et al. 2003, Bahia et al. 2002) can lead to differences in ER levels that cause modulation in AhR responsiveness. To prove that the effect of these estrogens on AhR activation depends

on cell context, several studies used ICI 182,780 to eliminate the effects of ERα. Similar to our studies, Gong et al. showed that ICI 182,780 and siERα increased basal levels of both *CYP1A1* and *CYP1B1*, suggesting that even unliganded ERα suppressed constitutive AhR activation. ICI 182,780 also reversed downregulation of *CYP1A1* by 10 nM (Gong et al. 2016) and 100 pM concentrations of E_2 (Wagner, Jiang, and Lehmann 2008). Gong et al. also showed that siERα potentiated GN or DZ (1 μM) induced *CYP1A1* and *CYP1B1* expression in MCF-7 cells; ICI 182,780 (1 μM) did not have a significant effect on either gene (Gong et al. 2016). However, similar to this study, ICI 182,780 did reverse GN (1 μM) and DZ (10 μM) downregulation of *CYP1A1* expression in MCF-7 BUS cells (Wagner, Jiang, and Lehmann 2008) and TCDD-induced *CYP1A1* expression (Marques, Laflamme, and Gaudreau 2013). In the current study, ICI 182,780 (1 μM) potentiated TCDD-induced *CYP1A1* and *CYP1B1* expression (Figure 7C, Figure 7D) and also increased *CYP1A1* expression after isoflavone treatments (Figure 7A, Figure 7C).. Additionally, after MCF-7 cells were pretreated with ICI 182,780, all isoflavones significantly induced XRE-luciferase activity in MCF-7 cells (Figure 8B), No significant induction in XRE-luciferase reporter activity was observed with the isoflavones alone in MCF-7 cells (Figure 8B), in which XRE reporter activity is usually lower compared to other cell lines, such as HepG2 and Hepa-1 cells (Wang et al. 2016a, Zhang, Qin, and Safe 2003).

In conclusion, the study showed that ERα agonists suppressed AhR activation in MCF-7 cells. An epigenetic mechanism specifically targeting *CYP1A1* was most likely the reason that RCE decreased 2-hydroxylation (*CYP1A1*), which led to a greater

increase in the 4-hydroxylation pathway in MCF-7 cells. Although BA and FN increased estrogen metabolism, it is important to note that they are not bioavailable and are rapidly metabolized to GN and DZ *in vivo* (Dietz et al. 2016, Piersen et al. 2004, Peterson et al. 1998). The effects of these phytoestrogens on estrogen metabolism are notable, especially because the 2-hydroxylation pathway is strongly associated with a decrease in breast cancer risk (Sampson, Falk, Schairer, Moore, Fuhrman, Dallal, Bauer, Dorgan, Shu, Zheng, et al. 2017). Modulation of this benign pathway may vary in women based on several factors such as ER status. Furthermore, as women are turning to BDSs for relief of menopausal symptoms during breast cancer treatments (i.e., tamoxifen, aromatase inhibitors) (Kligman and Younus 2010), it could be necessary to advise women with cancer against the consumption of certain BDSs containing isoflavones that induce the undesirable estrogen 4-hydroxylation pathway. Considering the *in vitro* nature of the available evidence, clinical studies are warranted that evaluate the corresponding safety parameters in these populations versus healthy women before recommendations about the clinical safety and population specificity of these BDSs can be made.

4 3D-EROD AS A MEASUREMENT OF P450 1A1/1B1 ACTIVITY

4.1 Rationale

4.1.1 Botanical extracts and estrogen chemical carcinogenesis:

In chapter 3, a baseline of isoflavone activity on estrogen oxidative metabolism was determined. However, the activity assay used in that investigation (LC-MS/MS measurement of $MeOE_1$ metabolites) required a high concentration of E_2 for the creation of a measurable concentration of these metabolites. Since the breast has an estrogen-rich local environment (van Landeghem et al. 1985) in both pre- and postmenopausal women (Yaghjyan and Colditz 2011), an activity assay that could measure overall changes in P450 1A1 and 1B1 activity in the presence of different concentration of estrogens needed to be created.

4.1.2 7-ethoxy-resorufin-*O*-deethylase assay issues:

The 7-ethoxy-resorufin-O-deethylase (EROD) was modified to fit this role. An already existing version of this assay (EROD monolayer; chapter 2) could detect AhR-mediated changes in P450 1 activity by measuring the conversion of 7-ethoxyresorufin by the P450 1 class of enzymes into the fluorescent resorufin in a kinetic in-cell assay (Dunlap et al. 2015a, Wang et al. 2016a). However, previous versions of the assay were limited in that ER-mediated P450 1A1 suppression could not be detected purportedly because of low basal P450 1A1 levels in MCF-7 cells (Spink, Spink, Cao, DePasquale, Pentecost, Fasco, Li, and R. 1998). (Figure 9).

4.1.3 **Mammospheres:**

Mammospheres are three dimensional spheroids composed of mammary cells that form a more realistic intratumoral environment than traditional monolayers (Maguire et al. 2016, Balachander et al. 2015, Haycock 2011, Lin and Chang 2008). Because spheroids express genes differently than monolayers of the same cell line (Maguire et al. 2016, Balachander et al. 2015), they were investigated for their potential ability to solve the issue of low *CYP1A1* expression in MCF-7 WS8 monolayer as well as providing a more realistic intercellular environment in which to conduct estrogen chemical carcinogenesis studies.

The aim of this study was to design a high-throughput EROD assay that can be used for the analysis of ER and AhR-mediated pathways, as well as to detect P450 1 inhibiting effects of both single compounds and complex mixtures, such as botanical extracts.

4.2 Results

4.2.1 **The 2D-EROD assay cannot distinguish between 1A and 1B1 activity.**

The influence of E_2 on *CYP1A1* and *CYP1B1* mRNA expression was first confirmed in MCF-7 monolayers (2D) by PCR. As previously reported (Marques, Laflamme, and Gaudreau 2013, Dunlap et al. 2017), E_2 treatment (1 nM, 24 h) significantly downregulated *CYP1A1* mRNA, but did not downregulate *CYP1B1* gene expression (Figure 9A). E_2-treatment did not significantly change overall P450 1 activity determined by the traditional EROD assay (Figure 9B). To address this problem, 3,5,2',4'-tetramethoxystilbene (TMS) a P450 1B1 selective inhibitor (Chun et al. 2001),

was added to the 2D-EROD assay (Figure 9C). Although only P450 1A activity was determined, the expected *CYP1A1* mRNA downregulation was not detected in the 2D-EROD assay **(**Figure 9C).

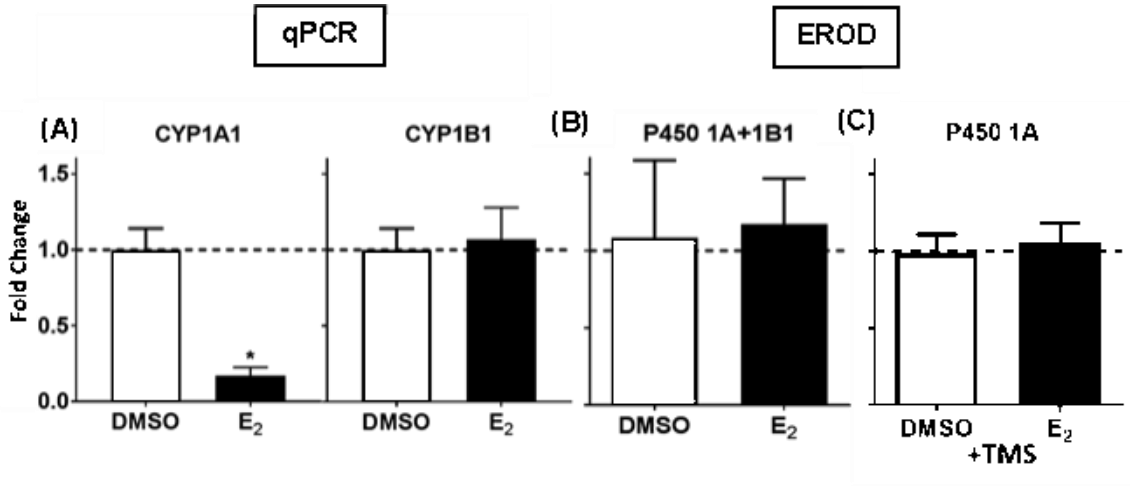

Figure 9: 2D-EROD cannot be used for ER mediated suppression of P450 1A1 with P450 1B1 selective inhibitor, TMS.
A) *CYP1A1*/*CYP1B1* gene expression and B) P450 1 activity in MCF-7 monolayer treated with negative control DMSO (0.1%) or estradiol (E_2; 1 nM; 24 h) measured by qPCR (A) or (B) 2D-EROD assay C) MCF-7 cells treated as in B, cotreated with 2,3',4,5'-tetramethoxystilbene (TMS; 1 µM). Normalized to and T-test compared to DMSO (A and B) or DMSO+TMS (C).

4.2.2 **Increased CYP 1A1 and *CYP1B1* expression and P450 1 activity in mammospheres:**

Because MCF-7 cells have low basal P450 1A1 levels and mammospheres have differences in mRNA expression to monolayers (Maguire et al. 2016, Balachander et al. 2015), we investigated the difference in mRNA expression in mammosphere (3D) vs monolayer (2D) MCF-7 models. *CYP1A1* and *CYP1B1* mRNA expression, but not *AHR* nor the mRNA for ERα encoding ESR1 were upregulated in 3D mammospheres (Figure 10A). To confirm this upregulation, the EROD activity assay was performed in 3D mammospheres. The signal to noise ratio was significantly increased in the 3D model (Figure 10B).

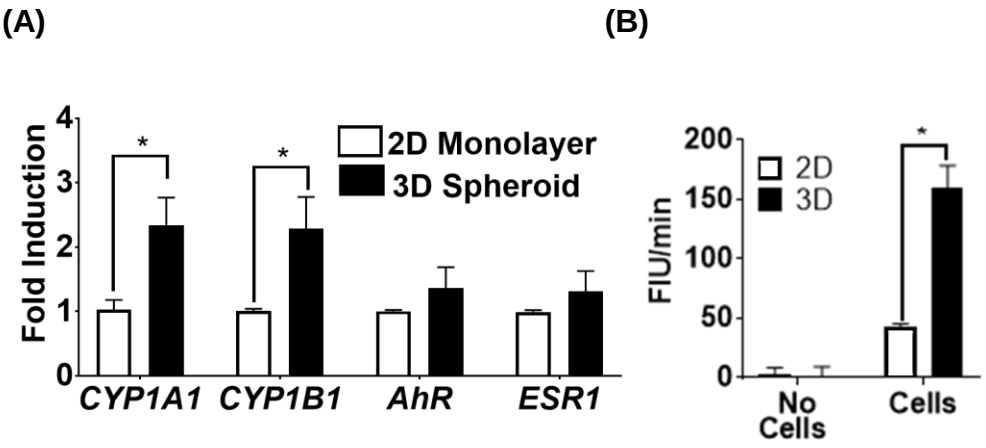

Figure 10: Increased *CYP1A1*/1B1 gene expression and P450 1 activity in 3D-mammospheres.
A.) *qPCR* of *CYP1A1, CYP1B1, AHR,* and *ESR1* gene expression in MCF-7 mammospheres and monolayers treated with DMSO (0.1%) for 24 h in MCF-7 cells. Compared to respective 2D monolayer gene expression. B.) EROD measurement of salicylamide, 7-ethoxyresrufin with (cells) and without (no cells) in monolayer (2D) or mammosphere (3D) with background (salicylamide + 7-ethoxyresirufin subtracted). (A) normalized to monolayer treated with DMSO (0.1%). T-test compared 3D model to corresponding 2D model.

4.2.3 **Adding TMS to mammosphere model treated with E_2 selectively downregulates P450 1A activity.**

When TMS was added to this 3D model, a significant decrease in P450 1A activity upon treatment with the ER agonist, E_2, was determined at 1 µM TMS (Figure 11A). While this TMS-EROD-3D model significantly decreased P450 1A activity at 1 nM E_2, the overall 1A/1B1 activity was not significantly influenced (Figure 11B). This confirmed the results observed by qPCR (Figure 9). The mammosphere EROD assay was also validated with the positive control for AhR mediated induction with AhR agonist

TCDD (10 nM; 24 h). The addition of TMS did not influence the ability to induce 1A by TCDD (Figure 11C) in mammospheres. This effect was confirmed with qPCR measurement of mammospheres treated with TCDD, both *CYP1A1* and *CYP1B1* mRMA expression was increased significantly (Figure 11D).

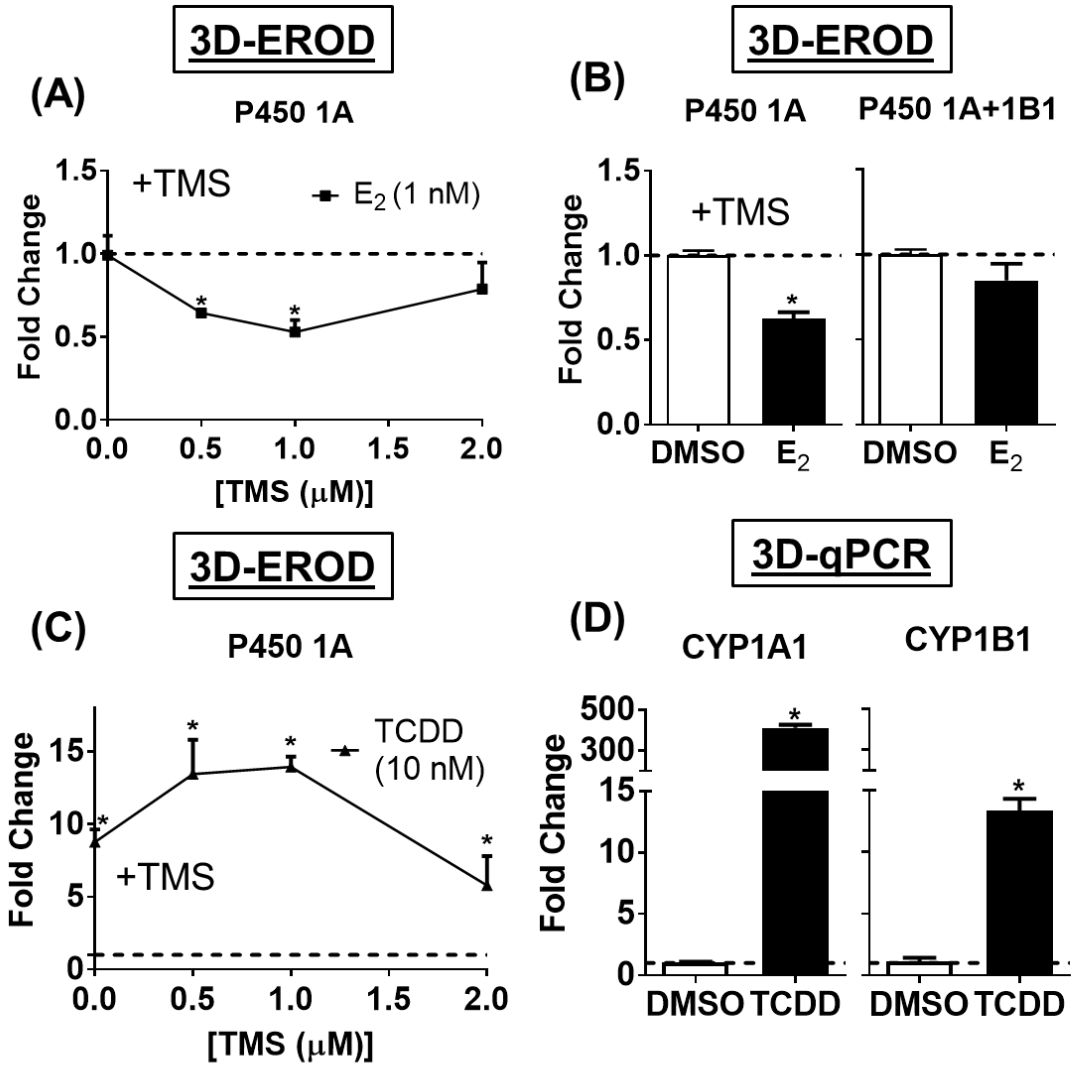

Figure 11: EROD in spheroids with TMS allows measurement of ER-mediated P450 1A1 inhibition without affecting measurement of AhR mediated increase of P450 1A in MCF-7 mammospheres.

P450 1 activity of cells cotreated 24 h with TMS (0, 0.5, 1, 1.5, or 2 μM) and a constant dose of negative control, DMSO (0.1%) or ER agonist, estradiol (E_2; 1 nM) and read with the same dose of TMS as added in cotreatment. B) P450 1 activity of cells treated and analyzed as in A but with 0 (P450 1A+1B1) or 1 μM TMS (P450 1A) and DMSO (0.1%) or E_2 (1 nM). C) P450 1 activity of cells treated the same as in (A) but with a constant dose of TCDD (10 nM) and changing TMS. D) qPCR measurement of *CYP1A1*/*CYP1B1* gene expression in mammospheres treated with TCDD (10 nM). Normalized to and T-test compared to respective dose of TMS (A, B, C) or DMSO (0.1%). *p<0.05.

4.2.4 **Control of cytotoxicity/proliferation by using the diameter to measure cytotoxicity**

Because the traditional methods of cytotoxicity such as MTT require a second plate of treated cells, mammosphere diameter was utilized as a control for cytotoxicity. Mammosphere diameter and MTT both quantified the expected results. E_2 should increase proliferation in these ERα+ spheres. In spheroids, a high dose of paclitaxel (10 µM) should be cytotoxic (Nicholson, Bibby, and Phillips 1997, Dong et al. 2013, Reynolds et al. 2017), and low dose (1 µM) should not be (Nicholson, Bibby, and Phillips 1997, Dong et al. 2013). E_2 treatment significantly increased both cell viability (MTT) and cell diameter. The low dose of paclitaxel did not significantly decrease cell viability nor diameter; the high dose of paclitaxel significantly decreased both cell viability and diameter (Figure 12).

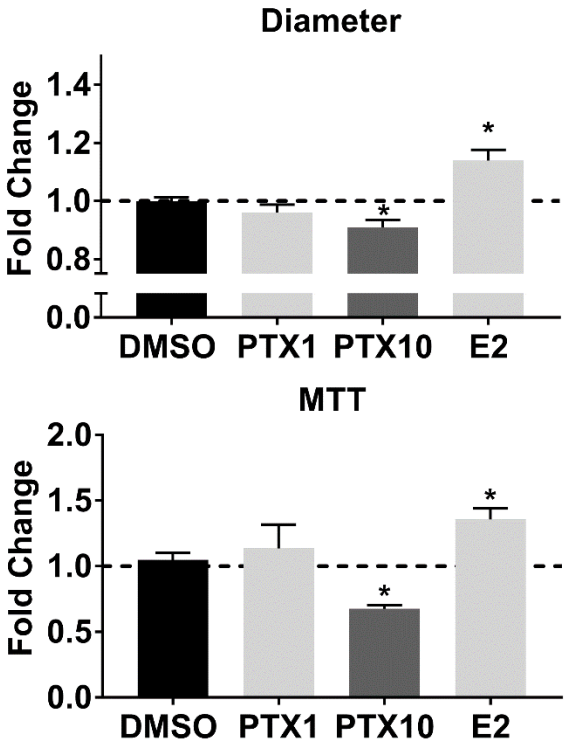

Figure 12: Mammosphere diameter is a measurement of cytotoxicity.
Sphere diameters or MTT readings of MCF-7 mammospheres treated with known cytotoxic (10 µM) and non-cytotoxic dose (1 µM) of paclitaxel (PTX), a known growth inducing dose of E_2 (1 nM), or DMSO (0.1%). Normalized and T-test compared to DMSO negative control.

4.2.5 **Validation of the assay: Controls yield expected dose-responsive results**

The AhR agonist, TCDD, dose responsively increased both P450 1A and P450 1A+1B1 activity in mammospheres. (Figure 13A). Treatment with AhR antagonist, CH 22191 (Company), dose responsively decreased both P450 1A and P450 1A+1B1 (Figure 13B). The E_2 dose response showed the expected selective decrease of the P450 1A pathway (Figure 13C), while the ERα antagonist, ICI 182,780, selectively increased P450 1A activity (Figure 13D). Bergamottin, a P450 1 inhibitor and weak AhR agonist first slightly increased AhR-mediated P450 1A + 1B1 activity then dose responsively directly inhibited 1A and 1B1 (Figure 13E). Mammospheres treated with TCDD and E_2 (10 nm; 1 nM) had significantly less P450 1A induction than those treated with TCDD alone (Figure 13F). All IC_{50}s/EC_{50}s of controls were in the pM-nM range with (Table I)

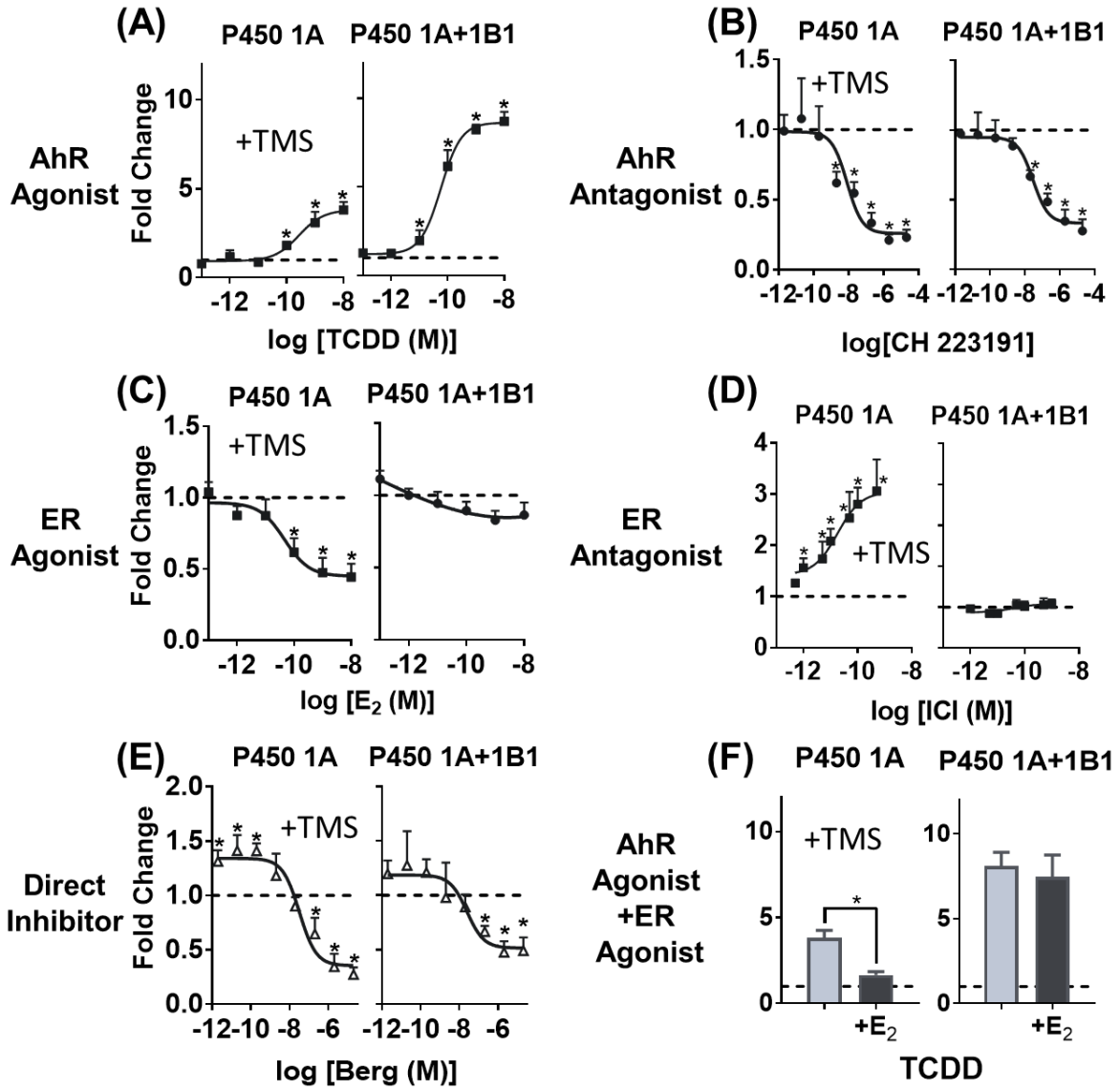

Figure 13: Validation of the 3D-EROD assay using potent controls demonstrating determination of multiple pathways on P450 1 activity.
Dose response of A) AhR agonist, TCDD; B) ER agonist, E_2; C) AhR antagonist CH 223191, D) ER antagonist ICI 182 780 E) P450 inhibitor bergamottin or F) 10 nM TCDD with or without TMS (1 μM). Normalized and T-test compared to DMSO+TMS (P450 1A) or DMSO (P450 1A+1B1) negative control.

.

TABLE I: IC_{50}/EC_{50}'S OF CONTROLS: MEANS ± SD DETERMINED FROM IC_{50}/EC_{50}'S OF THREE SEPARATE EXPERIMENTS IN EROD FIGURE 13.

	IC_{50} P450 1A (nM)	**IC_{50} P450 1A+1B1 (nM)**
TCDD	0.392 ± 0.3089	0.0806 ± 0.06506
CH 223191	93.5 ± 120.9	71.7 ± 101.9
E_2	0.0175 ± 0.01782	NA
ICI 182,780	0.0434 ± 0.03847	NA
Bergamottin	36.3 ± 8.143	27.5 ± 26.08

4.2.6 **Well-studied compounds and extracts yield expected results when screened with 3D-EROD:**

To confirm that 3D-EROD can evaluate modulation of oxidative estrogen metabolism, phytoconstituents and well-characterized extracts with known effects on estrogen oxidative metabolism were studied (TABLE III). Treatment with GN, an ER agonist, yielded the expected decrease in P450 1A activity. Treatment with biochanin A, an AhR agonist with estrogenic activity due to its metabolism in cells to GN, produced signs of both AhR and ER activity, with a decrease in P450 1A activity at low (2 µM) concentration and an increase in P450 1A+1B1 activity at high (20 µM) concentration. 6-PN, primarily an AhR agonist, significantly increased P450 1A and P450 1A+1B1 activity like the TCDD control (Figure 13A). Licochalcone A (LicA), an AhR antagonists,

decreased both P450 1A and P450 1A+1B1. (Figure 14;TABLE II: BIOLOGICAL ACTIVITIES OF COMPOUNDS AND EXTRACTS.).

TABLE II: BIOLOGICAL ACTIVITIES OF COMPOUNDS AND EXTRACTS.

	AhR agonists/antagonist	**ERα Agonists/Antagonists**	**P450 1A1 direct inhibition (Y/N)**	**P450 1B1 direct inhibition (Y/N)**
Genistein	NA	Agonists (Overk, Yao, Chadwick, Nikolic, Sun, Cuendet, Deng, Hedayat, Pauli, and Farnsworth 2005)	Y (Dean W. Roberts 2004)	Y (Dean W. Roberts 2004)
Biochanin A	Agonists (Han, Kim, and Jeong 2006b, Dunlap et al. 2017)	Agonist (Dunlap et al. 2017)	Y (Dean W. Roberts 2004)	Y (Dean W. Roberts 2004)
LicA	Antagonists (Dunlap et al. 2015b)	NA	NA	NA
6PN	Agonist (Wang et al. 2016a)	NA	NA	NA

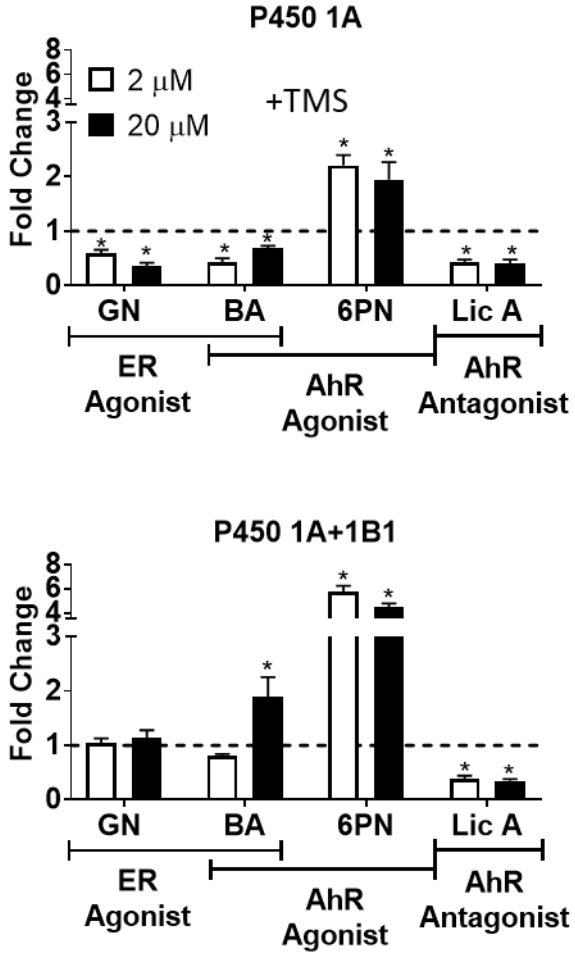

Figure 14: 3D-EROD can measure the overall effects of phytoconstituents with known activities.
MCF-7 mammospheres treated with GN, BA, 6-prenylnarigenin (6-PN), or licochalcone A (LicA), (2 or 20 μM) with (P450 1A) and without (P450 1A+1B1) TMS (1 μM) measured by 3D-EROD. Normalized to and T-test compared to DMSO alone (P450 1A+1B1) or DMSO+TMS (P450 1A). *p<0.05.

4.2.7 **XRE-luciferase reporter activity increased in MCF-7 WS8 cells treated with Bergamottin and TMS.**

In the course of designing the 3D-EROD assay, we ran into some unanswered questions. At low concentrations, bergamottin increased P450 1A activity (Figure 13E). Moreover, not cotreating with TMS and only adding it during analysis led to wide variations in readings (data not shown). Since TMS is an analogue of resveratrol, a known AhR agonists, it and bergamottin were tested for increased XRE-gene transcription, an indication of AhR activity (Figure 2). Both TMS and bergamottin significantly, yet weakly, increased XRE gene transcription with a decreased effect as concentration of treatment was increased. This effect made the finding that both very strong (TCDD; Figure 13A) and less strong (6-PN; Figure 14) AhR agonists still exert a significant change in activity particularly important.

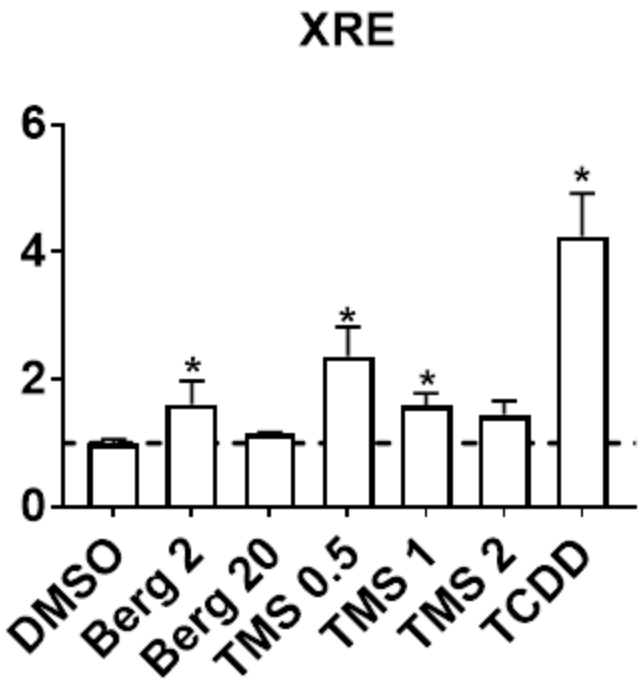

Figure 15: XRE-luciferase reporter activity increased in MCF-7 WS8 cells treated with Bergamottin and TMS.
Cells were treated with DMSO (0.1%), bergamottin (Berg; 2, 20 μM), 3,5,2',4'-tetramethoxystilbene (TMS, 0.5, 1, or 2 μM) or TCDD (10 nM). Normalized to DMSO negative control. T-test of compound compared to negative control *p<0.05.

4.3 Discussion:

4.3.1 Botanical Extracts and Estrogen Oxidative Metabolism:

Estrogen chemical carcinogenesis is one of many factors contributing to breast cancer risk. In estrogen chemical carcinogenesis, This process classically regulated by the AhR, which, when activated, upregulates P450s 1A1 and 1B1 and epigenetically

regulated by ERα, which selectively suppresses P450 1A1 (van Breemen et al. 2014). Single compounds and extracts can produce multiple effects on these pathways. For example, BA, a compound found in red clover is metabolized in breast cancer cells by P450 1A1 and P450 1B1 into GN (Roberts et al. 2004). GN directly inhibits P450 1A1 and 1B1 (Roberts et al. 2004) and acts as an ERα agonist (Overk, Yao, Chadwick, Nikolic, Sun, Cuendet, Deng, Hedayat, Pauli, and Farnsworth 2005).

Botanical supplements are taken by many women and contain thousands of compounds, multiples of which may affect multiple pathways in the estrogen chemical carcinogenesis pathways (Liu et al. 2013, Gertsch 2011) (chapter 3). Further, some of these compounds may interfere with the estrogen chemical carcinogenesis pathway. For example, BA, a compound found in red clover is metabolized in breast cancer cells by P450 1A1 and P450 1B1 into GN (Roberts et al. 2004). GN directly inhibits P450 1A1 and 1B1 (Roberts et al. 2004) and acts as an ERα agonist (Overk, Yao, Chadwick, Nikolic, Sun, Cuendet, Deng, Hedayat, Pauli, and Farnsworth 2005). Thus, having a simple assay to screen for changes in the overall effect of a compound or extract on the activity of P450 1A1 and 1B1 is of paramount importance.

4.3.2 **EROD in the past and MCF-7 WS8 cells:**

The EROD assay has been extensively utilized to monitor for AhR agonists because of the metabolism of 7-ethoxyresorufin by the P450 1 family of enzymes into the fluorescent resorufin (Petrulis et al. 2001). A kinetic assay was previously used by our lab to monitor overall P450 1A1 and 1B1 activity as a measure of AhR agonists or antagonistic activity (Dunlap et al. 2015b, Wang et al. 2016a). However, its usefulness

in measuring P450 1A1/1B1 activity has been limited because of its inability to separate the AhR mediated increase of P450 1A1/1B1 from the ERα mediated P450 1A1 suppression (Figure 9).

The breast cancer MCF-7:WS8 cell line was chosen as a model for estrogen chemical carcinogenesis in breast tissue, because these cells are highly responsive to both estrogen-mediated (Maximov et al. 2010, Sweeney, Fan, and Jordan 2014) and AhR-mediated effects (Callero and Loaiza-Pérez 2011, Al-Dhfyan, Alhoshani, and Korashy 2017). Thus, the EROD assay was modified for MCF-7:WS8 cells to screen for changes in P450 1A/1B1 activity.

4.3.3 **Issues with EROD:**

EROD in MCF-7:WS8 monolayer was unable to quantitate the ER-mediated P450 1A1 inhibition seen by qPCR measurement of *CYP1A1* in MCF-7:WS8 monolayers treated with E_2, purportedly due to the inability of 7-ethoxyresorufin to distinguish between enzymes in the P450 1 family enzymes. To separate ERα-mediated selective *CYP1A1* downregulation from AhR-mediated *CYP1A1*/1B1 upregulation, a P450 1B1 selective inhibitor was used.

4.3.4 3,5,2',4'-Tetramethoxystilbene**:**

3,5,2',4'-Tetramethoxystilbene: (TMS) was first chosen because of its easy availability and multiple papers referring to its selective P450 1B1 inhibiting properties (Chun et al. 2001). It was first added immediately before running the analysis; however, results were inconsistent (data not shown). TMS itself did not significantly affect kinetic fluorescence reading (data not shown). Because of its structural similarity to another

AhR agonists, resveratrol and a report of increased *CYP1A1* expression (Einem Lindeman, Poirier, and Divi 2011), the effect of TMS on XRE gene transcription was measured (Figure 15). It was found to be a likely weak AhR agonist (Figure 15). The difficulties achieving consistent results when TMS was only added for during analysis may have been due to this AhR mediated effect. Thus, co-treatment with TMS (1 µM) was added to the method and the effect of TMS in the experiment appeared to be dual; there is a slight increase in AhR mediated activity followed by selective direct inhibition of P450 1B1. This approach not only yielded the expected ER-mediated suppression of both with treatment with a strong estrogenic compound (E_2) (Figure 11; Figure 13), and with the weaker phytoestrogen, GN (Figure 14). Moreover, it did so without removing the ability to capture the AhR agonistic effect of increased P450 1A1 activity both with the strong AhR agonist, TCDD, (Figure 13) as well as weaker agonist, 6-PN (Figure 14).

4.3.5 **Mammospheres/spheroids:**

Mammospheres are three dimensional spheroids composed of mammary cells with a myriad of advantages over traditional 2D-monolayers. Spheroids' genomic profiles stay more similar to the primary culture compared to monolayers (Balachander et al. 2015, Maguire et al. 2016, De Witt Hamer et al. 2007). Likewise, cells in spheroids function more similarly to tissue than 2D-monolayers (Gaskell et al. 2016, Haycock 2011, Lin and Chang 2008, Raghavan et al. 2016, Mehta et al. 2012, Correia and Bissell 2012).

Although spheroids are used in few activity assays , they are used in EROD assays performed in liver cells (Nakazawa et al. 2006, Cipriano et al. 2017, Sakai et al.

2010, Wu et al. 1999) and to understand structural changes in carcinogenesis (Haycock 2011, Raghavan et al. 2016, Friedrich et al. 2009). MCF-7 WS8 cells readily form structurally similar spheres when placed in rounded low adherence plates (data not shown).

4.3.6 **Salicylamide:**

Our previous in-cell 2D-EROD kinetic assay, used salicylamide was used to prevent the formation of secondary metabolites and to prevent the degradation of resorufin and thus the degradation of the fluorescence signal (Wang et al. 2016a). This was found to be important in spheres as well; assays without salicylamide yielded curves rather than straight lines (results not shown).

4.3.7 **Treatment size and time:**

The specific treatment time was selected because of the prevalence of the 24-hour time point in the various experiments in literature to which these results are compared (Lai, Wong, and Wong 2004, Marques, Laflamme, and Gaudreau 2013). Since this assay can measure P450 1A and P450 1A+1B1 activity, it should be able to quantitate effect at other time points as well. However, we found that it is important to keep final sphere diameter between 500 and 1000 µM (Friedrich et al. 2009). The necrotic core does not consistently form spheres smaller than 400 µM and we found that cells began to give inconsistent results below that diameter (data not shown). Although literature varies on the upper limit of sphere size, we found that the spheres started to lose consistency of shape and reproducibility around 1000 µM. Between

those sizes, data as measured by fold change from negative control were consistent making this assay robust to small counting errors.

4.3.8 **Polypharmacology of Bergamottin:**

Bergamottin's initial increase of P450 1A and P450 1A+1B1 activity was found to be possibly due to AhR mediated effects as it activated XRE gene transcription in MCF-7:WS8 cells (Figure 15). One study tested for AhR agonist activity without the presence of other AhR agonists and found no increase of activity, but it was done in mouse cells (Baumgart et al. 2005). To our knowledge, the bergamottin has not been tested for AhR activity in MCF-7 WS8 cells without the presence of other stronger, AhR agonists.

4.3.9 **Dose responses compared to literature:**

To our knowledge, there have been no dose response studies of the control compounds in mammospheres, so IC_{50}/EC_{50}s were compared to those in monolayers. It is, therefore, expected that the IC_{50}/EC_{50}s in this assay would vary from those in literature which used monolayers cultures. Only one compound differed widely from literature with the EC_{50} of TCDD in 3D-EROD falling in the pM rather than low µM range (Li et al. 2014) while the IC_{50} of CH 223191's IC_{50} concurred with literature values in the mid-µM range (Kim et al. 2006), which could be due to the a difference in cell signaling in mammospheres when compared to monolayers. In this 3D-EROD assay, E_2's ERα mediated effects in MCF-7:WS8 cells were found to have EC_{50}s in the pM range (Yang et al. 2014) and ICI 182,780's EC_{50} fell in the pM range (Yang et al. 2014). Bergamottin's IC_{50}s in intact cells varied greatly, ranging from the low nM to low µM range (Baumgart et al. 2005, Kleiner, Reed, and DiGiovanni 2003). Our findings fall

within the lower end of those found in literature. Thus, all of those effects can be measured with this 3D-EROD assay which can capture an overall picture of estrogen oxidative metabolism by separating ERα-mediated activity from AhR-mediated activity as well as capturing direct inhibition of P450 1A1 and 1B1 (Figure 13E). Since the 3D-EROD assay can capture the overall effects of botanical extracts and compounds on estrogen oxidative metabolism in a 96-well format which can be easily adapted to robotics, it can be utilized for high-throughput screening of a myriad of potential drugs and extracts for potentially harmful or beneficial effects in cancer prevention.

4.3.10 **Complications and Limitations of 3D-EROD:**

The use of mammospheres may complicate our understanding of modulation due to their added complexity. They are known to be differently affected by treatments in different part of the sphere (Reynolds et al. 2017). As this assay stands, it cannot distinguish where in the spheroid the changes in activity are occurring.

Moreover, this assay only measures P450 1A/1B1 activity. It does not capture anything occurring in estrogen oxidative metabolism after that point such as modulation of COMT. Additionally, it cannot separate the cause of the modulation. For example, it cannot determine if P450 1A/1B1 inhibition is due to an AhR antagonist or a direct inhibitor of P450 1 family enzyme (Figure 13).

5 EVALUATION OF WOMEN'S HEALTH EXTRACTS

5.1 Rationale and hypothesis

The UIC/NIH Center for Botanical Dietary Supplements Research, in which this work was completed, focuses on better understanding compounds and extracts used for women's health. While we work on improving our understanding of biological mechanisms and creating better model systems in which to measure biological effects, we also evaluate extracts and their bioactive compounds to decide which are worth further investigation. In this chapter we seek to utilize the assay designed in the previous chapter as well as computational modeling and an assay to measure biological activity to design innovative approaches to answer question related to extracts studied in the botanical center. This chapter outlines a few examples from projects that were halted as well as the investigation into how hydrolysis affects activity and which extract was chosen for further study in the next chapter.

The extracts studied can contain thousands of compounds, and compositions can vary dramatically (Uifălean et al. 2015). This complexity was tackled by three main approaches: screening different extracts from the same plant, bioassay guided fractionation, and computational modeling.

5.1.1 Extract screening:

Extracts can not only vary dramatically in chemical composition (Uifălean et al. 2015), but the chemical composition of the extract often varies markedly from the form of the active compounds in the body. For instance, soy extracts often contain phytoestrogens in their estrogenically inactive glycosylated form rather than their

biologically active aglycone. With the 3D-EROD assay developed in chapter 4 and the alkaline phosphatase assay in Ishikawa cells for estrogenic activity, the effects of hydrolysis on biological activities were investigated.

5.1.2 **Bioassay guided fractionation:**

Bioassay guided fractionation is a well-established method for narrowing down the active compounds in an extract. In this process, an active extract is separated into separate fractions which are then tested for biological activity. The active fraction(s) is/are further separated into subfractions until analysis of active fractions' compounds indicate individual compounds that should be tested for biological activity (Weller 2012).

5.1.3 **Computational modeling**

Docking involves computationally binding of a ligand into the ligand binding domain (LBD) of a receptor often downloaded from the Protein Data Bank's collection of crystal structures. It is an important in silico tool for investigating structure/activity relationships and predicting binding conformations. There are three major computational methods used for docking: lock and key, rigid receptor, and induced fit. Lock in key does not allow the LBD nor the ligand to move to find their most energetically favorable pose. Rigid receptor allows the ligand but not the receptor to move. Induced fit allows movement of both. One way of validating a model derived from a crystal structure that was crystalized with a ligand bound is to dock that same ligand and confirm that their conformations are similar (Forli et al. 2016).

In this study, the rigid receptor evaluation of ERα ligands could not be validated as it did not pass that test. Thus, induced fit modeling was utilized. However,

computational models, particularly induced fit models, are plagued with false positives (Chambert, Miller, and Nichols 2015). Since we are only investigating a few types of compounds, computational modeling as a screening technique is limitedly useful. Therefore, instead of using computational modeling to generate binding energies and use those energies to predict which compounds would be the best binder, we used them to better understand *in vitro* data to improve understanding about binding properties and use that understanding to predict which isoflavones may be responsible for activities.

With a combination of these techniques, hypotheses were generated for future work. A few selected examples are given in the Results/Discussion section. The separation and characterization of extracts were completed by members of Dr. Guido Pauli's lab in collaboration within the UIC/NIH Center for Botanical Dietary Supplements Research and the details of their chemical composition are not included in this chapter.

5.2 Results/Discussion:

5.2.1 Fatty Acid Fractions as probable aryl hydrocarbon receptor agonists:

5.2.1.1 Roseroot:

Roseroot (*Rhodiola rosea*) is a plant that grows in artic regions that is traditionally taken as an adaptogen. Recently, it has garnered attention as a treatment for alleviation of menopausal symptoms (Gerbarg and Brown 2016). The study outlined here shows an example of bioassay guided fractionation utilizing the assay from the previous chapter (3D-EROD).

3D-EROD screening of extracts from TABLE III: EXTRACTS SCREENED revealed that this methanolic roseroot extract (RR) increased both P450 1A and P450 1A+1B1 activity (Figure 16), an effect that an AhR agonist would typically show (Figure 16).

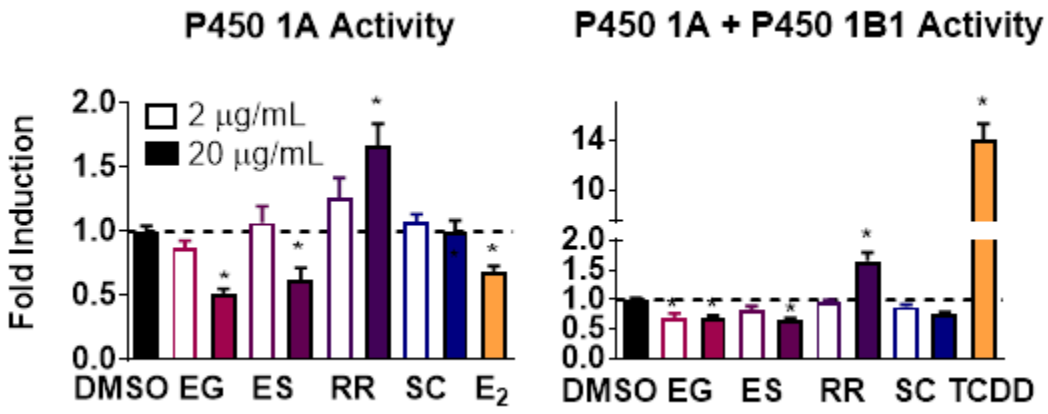

Figure 16: Screening revealed probable AhR mediated induction of P450 1A+1B1 activity by roseroot.
3D-EROD measurement of P450 1 activity in MCF-7 mammospheres treated for 24 hours with extracts defined in Table III or E_2 (1 nM) or TCDD (10 nM) with (P450 1A) or without (P450 1A+P450 1B1) TMS (1µM). Normalized and T-test compared to DMSO+TMS (P450 1A) or DMSO (P450 1A+1B1) negative control.
*p<0.05.
.

TABLE III: EXTRACTS SCREENED IN FIGURE 16

Sample:	Identity
EG	Horny goat weed (*Epimedium grandifolium*) MeOH extract
ES	Horny goat weed (*Epimedium sagittatum*) MeOH extract
RR	Roseroot (*Rhodiola rosea*) MeOH extract
SC	Five flavor berry (*Schizandra chinensis*) MeOH extract

Roseroot has at least two bioactive compounds which are often used for standardization: salidroside and rosavin (Chiang et al. 2015, Elameen, Dragland, and Klemsdal 2010). Neither those compounds nor the extract is known to affect estrogen oxidative metabolism. The roseroot extract was fractionated, and those fractions and those compounds were screened for AhR mediated P450 1A+1B1 activity. Neither salidroside nor rosavin significantly modulated AhR mediated induction or inhibition of P450 1A+1B1 activity (Figure 17). Both fractions 4 and 5 contained this active compound, with 5 more active than 4 (Figure 17). Fraction 5 was determined to contain primarily fatty acids (data not shown). Fatty acids can act as AhR agonists (Jin et al. 2017). As this is an activity assay in cells, it should not have false positives unless compounds are fluorescent. This was corrected for by the washing step in the 3D-EROD assay, and controls were run to confirm that there was no measurable

fluorescent interference (data not shown). However, this class of compounds was outside of the scope of the goals of our studies, and this work did not continue.

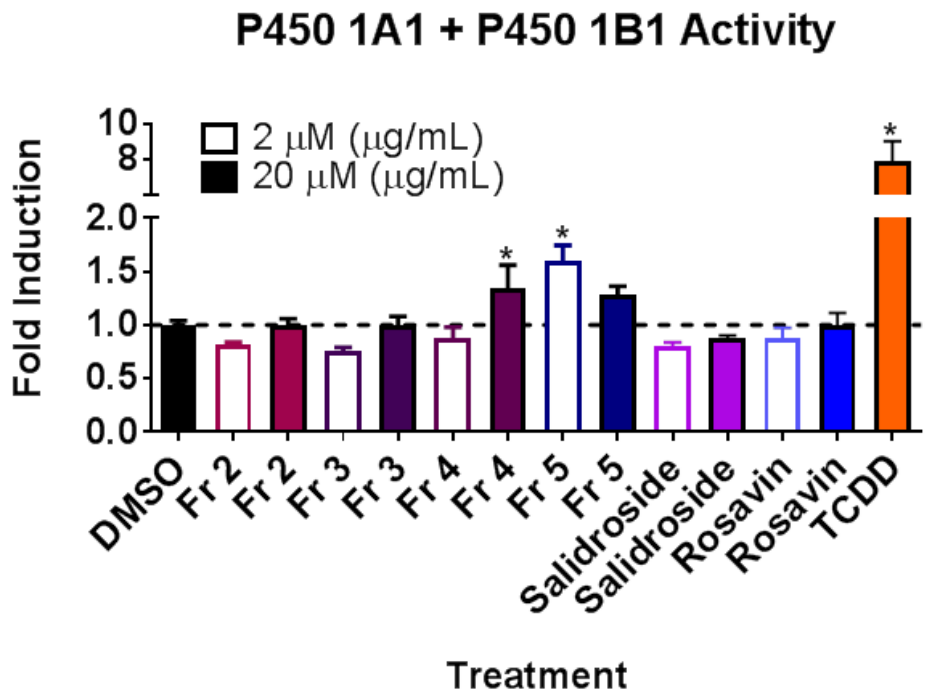

Figure 17: Probable AhR mediated induction of P450 1A+1B1 activity by roseroot fraction 4.
3D-EROD measurement of P4501 activity of MCF-7 mammospheres treated for 24 hours with fractions of roseroot or compounds or TCDD (10 nM). Normalized to and T-test compared to DMSO (0.1%). *p<0.05

5.2.1.2 Milk thistle (silymarin):

Silymarin *Silybum marianum* . (Milk thistle), has been used for centuries for liver disorders and female complaints. Silymarin is the active component of this herb, which

is a complex of components, mainly silybin A, silybin B, isosilybin A, and isosilybin B. It was investigated by the botanical center due to its use in women's health.

Milk thistle was found to, dose responsively, induce a presumably AhR mediated increase in P450 1A + 1B1 similar to that from treatment with TCDD (Figure 13). It did not increase P450 1A, thus indicating a preferential P450 1B1 induction (Figure 18).

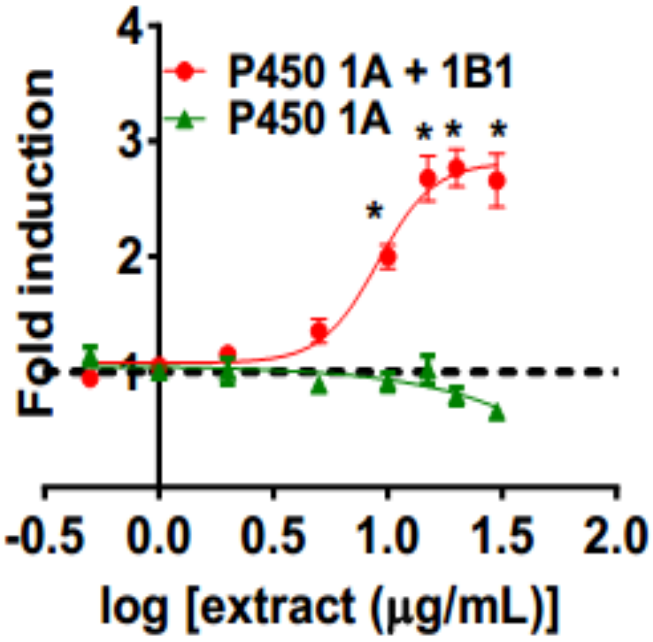

Figure 18: Silymarin has dose-responsive probably AhR mediated induction of P450 1A+1B1.
3D-EROD measurement of P4501 activity of MCF-7 mammospheres treated for 24 hours with milk thistle (silymarin) extract with (P450 1A) or without (P450 1A + 1B1) TMS (1µM). Normalized and T-test compared to DMSO+TMS (P450 1A) or DMSO (P450 1A+1B1) negative control. *p<0.05.

This milk thistle extract was fractionated, and those fractions were tested for induction of P450 1 activity. Fraction 21 was found to be the best inducer of activity. The major reported bioactive compound, silibinin, only showed activity at high concentrations (Figure 19)

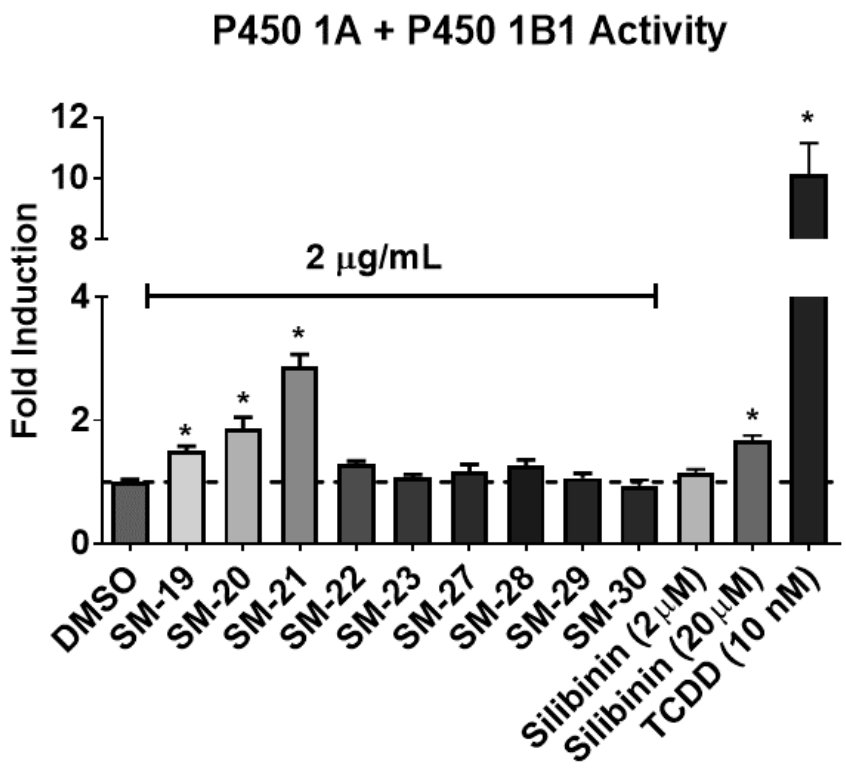

Figure 19: Silymarin fraction 21 has the most probably AhR activity.

Screening silymarin fractions: 3D-EROD measurement of P4501 activity of MCF-7 mammospheres treated for 24 hours with fractions of roseroot or compounds. Means + SEM; n=3. Student's T-test comparing treatments to DMSO (0.1%) negative control * $p<0.05$.

Fraction -21 from above was further fractionated into subfractions. Fraction 7 was the only fraction to increase P450 1 activity (Figure 20). Subfraction 19 contained primarily fatty acids (data not shown). This class of compounds was outside of the scope of our studies and this work did not continue.

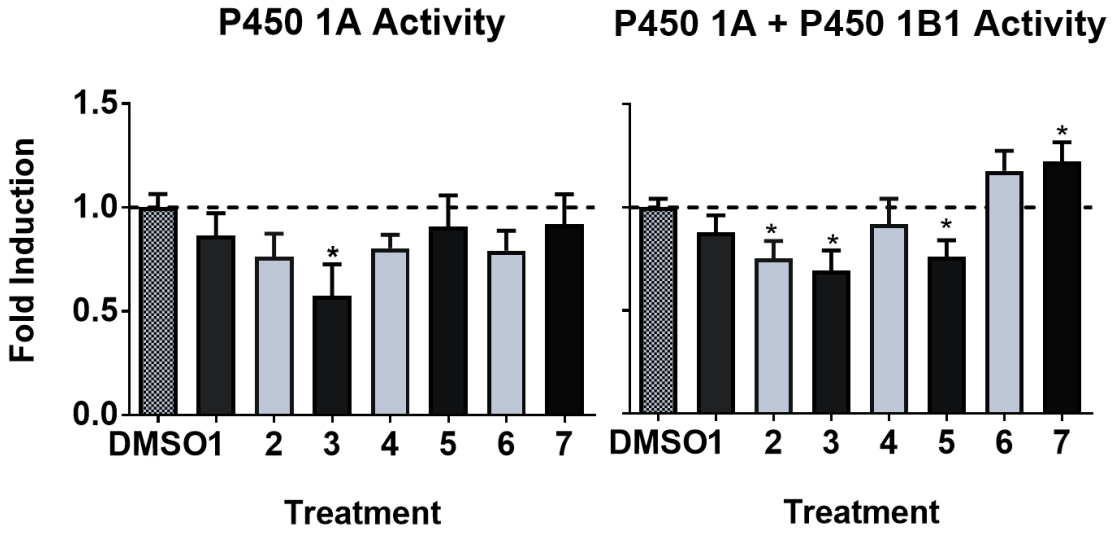

Figure 20: Silymarin subfraction 7 contains probably AhR agonist.
Screening of silymarin subfraction: 3D-EROD measurement of P4501 activity of MCF-7 mammospheres treated for 24 hours with subfraction (2 μg/mL) with (P450 1A) or without (P450 1A + 1B1) TMS (1μM). Normalized and T-test compared to DMSO+TMS (P450 1A) or DMSO (P450 1A+1B1) negative control. *$p<0.05$.

5.2.2 **Hydrolysis of soy bean extract yields increased estrogen mediated effects:**

Beyond the chemical complexity of botanicals, there lies another problem: *in vivo* metabolism leads to metabolites in the tissues that are not present in the plant extract (Setchell et al. 2001). In highly glycosylated extracts, such as soy bean, the extract can be hydrolyzed to form the biologically active aglycone: (Setchell 1998, Setchell et al. 2001). Studies of the effect of hydrolysis of extracts on their biological effects can start to bridge the gap between *in vitro* and *in vivo* studies. However, such studies require analysis of biological effects of multiple time points and/or hydrolysis methods.

This assay was utilized to screen a soy bean extract autohydrolyzed for 0, 2, 4, or 6 days. As confirmed by the alkaline phosphatase assay, estrogenicity and ERα-mediated P450 1A suppression was increased with hydrolysis time (Figure 21). Thus, 3D-EROD captured the estrogen mediated effects and indicated that hydrolyzed extracts significantly affected P450 1A activity while the unhydrolyzed extract did not.

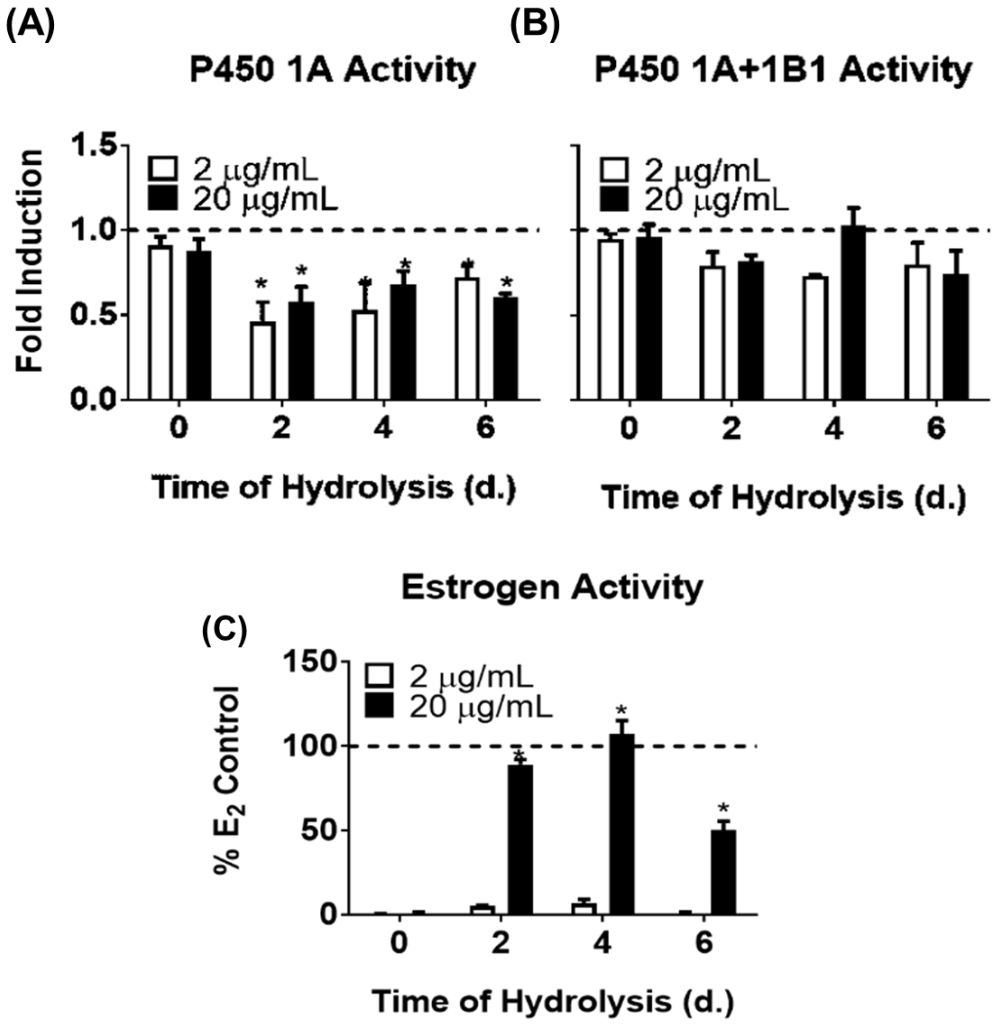

Figure 21: Hydrolysis increases biological activity.
3D-EROD measurement of MCF-7 monolayers treated with hydrolyzed or unhydrolyzed extracts made from soy beans with (A) and without (B) TMS. C) Induction of alkaline phosphatase in Ishikawa cells by soy extracts. Results were normalized to DMSO or $\%E_2$ (C) and are shown as fold induction. Results are the means of three independent determinations. Normalized and T-test compared to DMSO+TMS (A) or DMSO (B, C) negative control. *$p<0.05$.

5.2.3 **Computational modeling:**

Introduction sentence of what you like to analyze here. To ensure the fit of the positive controls, an induced fit model was used instead of a rigid receptor model in which the native conformation of GN from the crystal structure could not be reproduced (data not shown). Induced fit modeling is plagued with false positives, in part, because of the receptor moving in ways it may not be capable of moving and to the probability of the ligand making it into the binding pocket not being considered. Therefore, we took a different approach. Ligands were computationally docked into binding sites of enzymes we had already studied *in vitro*. Hypotheses were then formed to explain differences in binding affinity. These hypotheses can be used to guide further *in vitro* experiments and to help choose which compounds to first consider without going through as many rounds of bioassay guided fractionation. It is important to note that the differences in binding score, the measure used to predict the binding affinity, were small and are, therefore, not mentioned, and that in induced fit modeling, compounds will be shown to bind in the receptor even though they may not actually bind there. Below are some examples of how this tool was used.

5.2.3.1 *Z*-ligustilide estrogenicity:

Dong quai, the dried root of *Angelica sinensis*, is a common traditional Chinese medicine used to treat menstrual disorders such as dysmenorrhea (Chen et al. 2013). Whether dong quai has estrogenic activity is controversial (Piersen 2003, Hajirahimkhan, Dietz, and Bolton 2013b). *Z*-ligustilide is seen as one of the main biologically active compounds in Dong Quai. In various experiments, including

estrogenicity, our lab found wide variation in biological effects of both the extract and Z-ligustilide alone (data not shown). Since Z-ligustilide is unstable, computational modeling was utilized to see if the compound itself is likely to be responsible or whether other avenues such as i the degradation products should be explored.

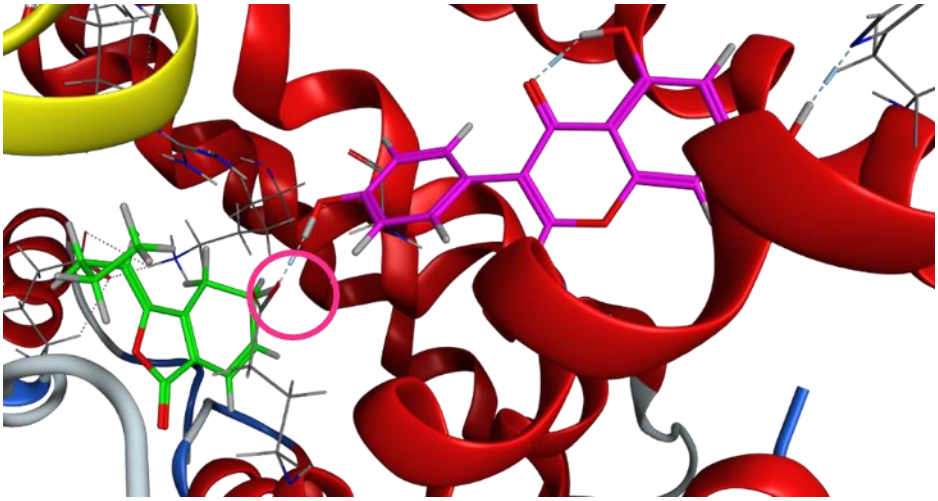

Figure 22: *Z*-ligustilide (green) does not bind to Glu (encircled) necessary for biological effect. *Z*-Ligustilide (green) and genistein (magenta) docked in binding pocket of ERα.

Although *Z*-ligustilide did bind to the LBD with a good binding score, it does not bind in the same location as phytoestrogens/E_2 (shown as GN for reference) nor did it bind to the encircled Glutamic acid (Glu) residue. When site-directed mutations were

introduced into LBD, enzymes with the Glu-> Lys mutation of this residue were unable to recognize the coactivator (Shiau et al. 1998). Moreover, the same Glu residue was found to be integral to strong antagonistic activity; it was at least in part responsible for 4-OH tamoxifen's strong antiestrogenic activity while tamoxifen did not strongly inhibit activity (Fang et al. 2001). These data support the hypothesis that *Z*-ligustilide itself is not responsible for the estrogenic or antiestrogenic activity, which it is sometimes reported to have, and indicates a need for more careful handling of unstable compounds for more consistent results.

5.2.3.2 Selectivity of isoflavones: ERα vs. AhR:

Structurally similar isoflavones may be selectively bind to ERα or AhR (Chapter 3). In the hop extract, the location of a prenyl group on a prenylated naringenin can dramatically effect binding affinity: 8-prenylnarigenin (8-PN) is an ERα ligand (Brunelli et al. 2007) but not a strong AhR ligand (Wang et al. 2016a), while 6-prenylnaringenin (6-PN) is an AhR ligand (Wang et al. 2016a), but not a strong ERα ligand (Overk, Yao, Chadwick, Nikolic, Sun, Cuendet, Deng, Hedayat, Pauli, Farnsworth, et al. 2005) .

Computational modeling revealed that, in the binding pocket of AhR, the hydrophobic prenyl group of the AhR agonist, 6-PN, is placed in a hydrophobic pocket (green). The non-AhR agonist 8-PN's prenyl group is forced towards a hydrophilic (purple) region and is crowded into an energetically less favorable position (Figure 23

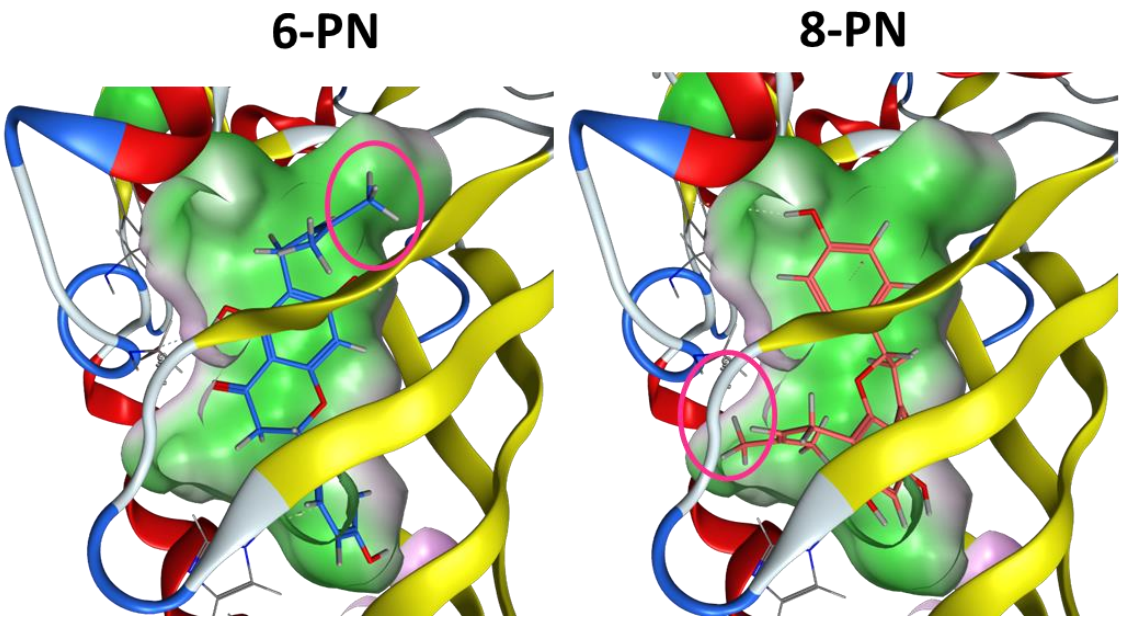

Figure 23: 6-PN's prenyl group in hydrophobic pocket; 8-PN's forced into hydrophilic region.
6-PN and 8-PN docked in the binding pocket of AhR. 6-PN and 8-PN docked in the LBD of AhR. Green regions represent hydrophilic regions; purple regions are hydrophilic.

On the other hand, in the ERα binding pocket, the opposite occurs; the hydrophobic prenyl group of the ER agonist, 8-PN, is placed in a hydrophobic pocket (green). The weak ER agonist 6-PN's prenyl group is forced into a hydrophilic (purple) region. Moreover, the positioning of the prenyl group forces the ring structure to crowd into an energetically less favorable position. (Figure 24)

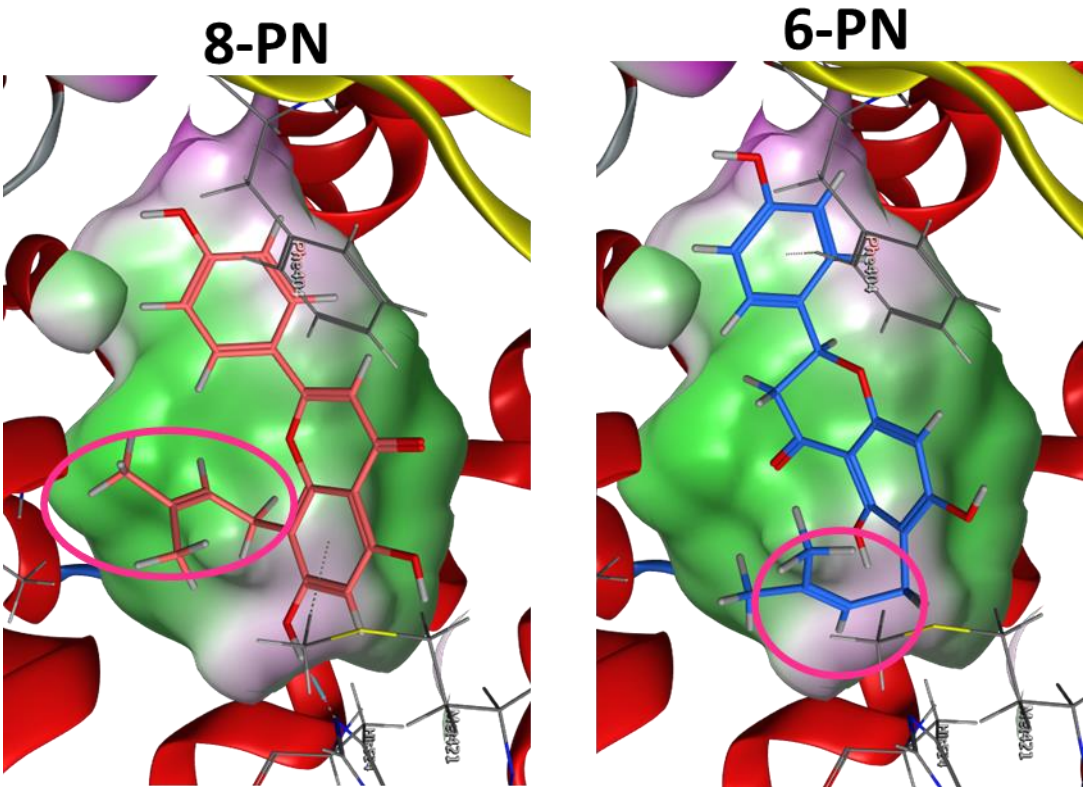

Figure 24: 8-PN's prenyl group in hydrophobic pocket and -OH group in hydrophilic region; 6-PN's forced into hydrophilic region in ERα binding pocket. 8-PN and 6-PN docked in the LBD of ERα. Green regions represent hydrophilic regions; purple regions are hydrophilic.
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In the red clover extract, the difference of one methoxy group (biochanin A; BA) vs a hydroxy group (GN) dramatically affected binding affinity. GN is an ERα ligand but not a good AhR ligand while BA is an AhR ligand but not a good ERα ligand (Chapter 3).

Computational modeling revealed that, in the binding pocket of AhR, the less hydrophilic methoxy group of the AhR agonist, BA, is placed in a hydrophobic pocket (green) near a hydrophilic region (purple). The non-AhR agonist GN's hydrophilic hydroxy group is forced into the hydrophobic (green) region, thus binds less favorably (Figure 25).

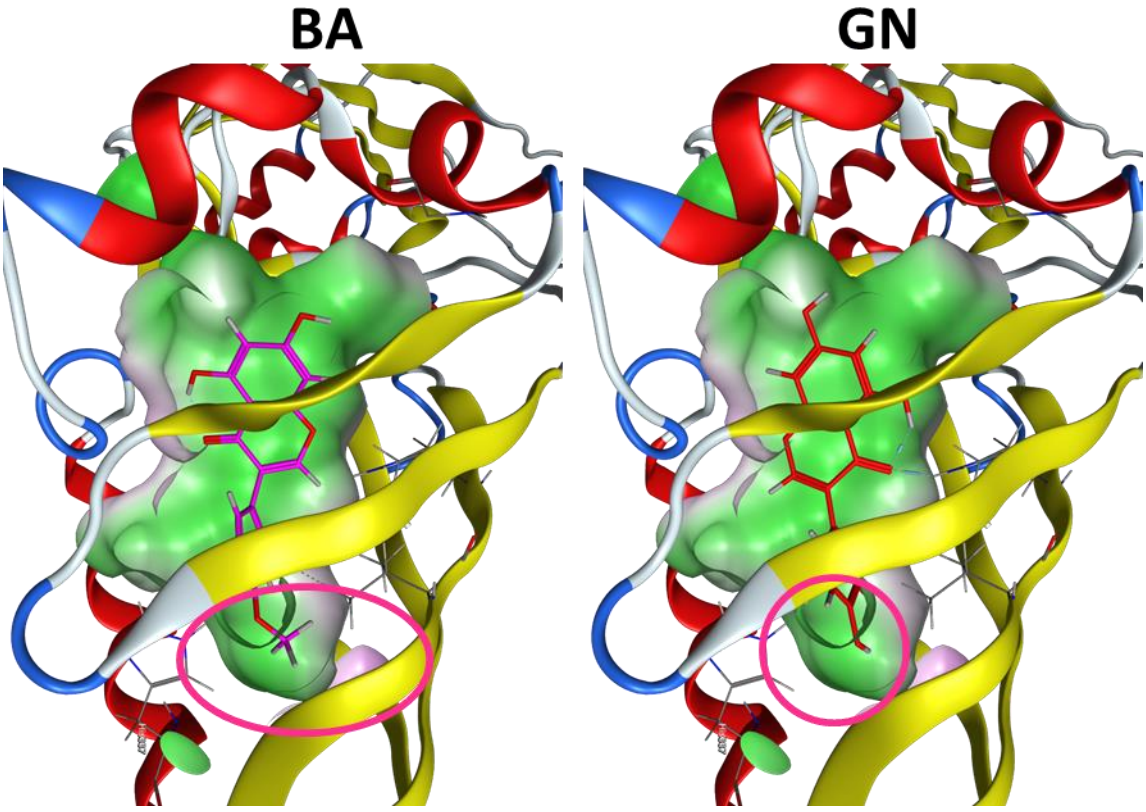

Figure 25: GN's hydroxy group falls in the hydrophobic region; BA's methoxy group lies in the hydrophobic region in AhR binding pocket.
GN and BA docked in the LBD of AhR. Green regions represent hydrophobic regions; purple regions are hydrophilic.

On the other hand, in the ERα binding pocket, the opposite occurs; the hydrophobic hydroxy group of the ER ligand, GN, is placed in a hydrophilic (purple) region. The non-ER agonist, BA's less hydrophilic methoxy group lies in a hydrophilic (purple) region. Moreover, BA preferentially binds in the less favorable direction from most estrogenic flavonoids as well as the opposite direction of genistein found in the crystal structure. Therefore, the hydrophobicity of the functional groups and the binding direction may be responsible for they different selective binding (Figure 26).

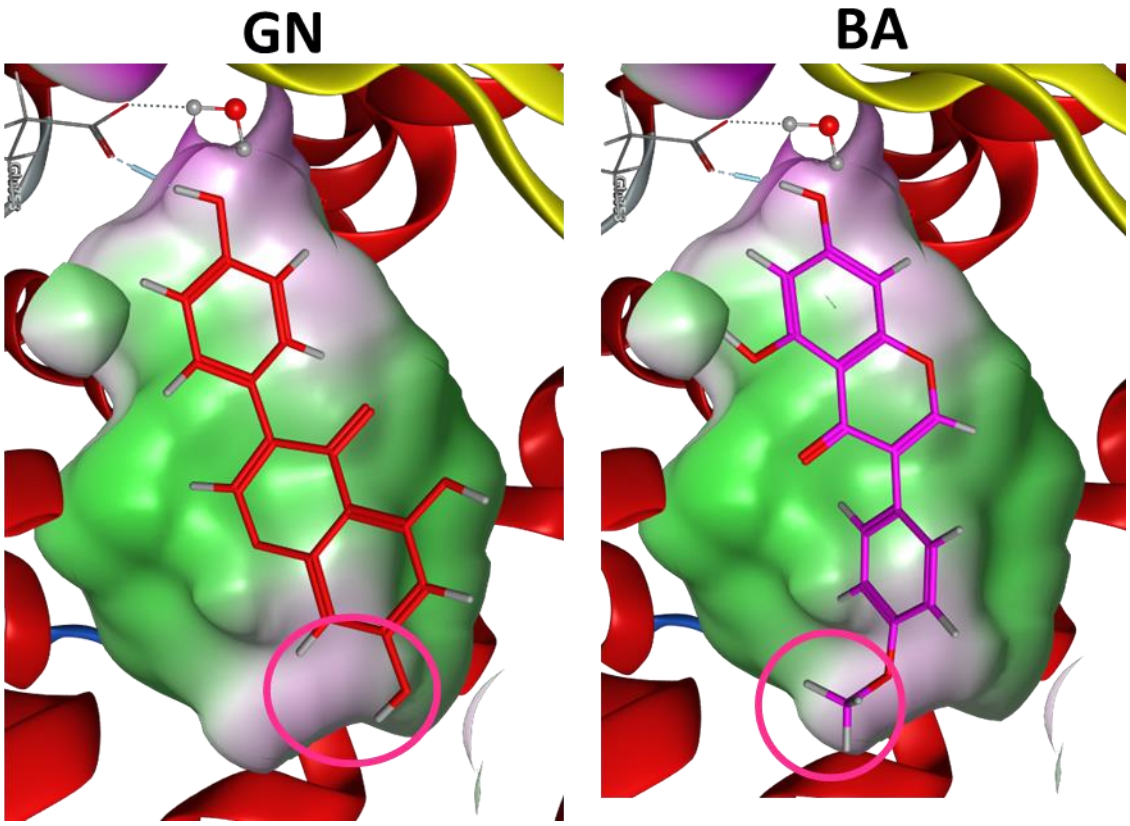

Figure 26: GN's hydroxy group fits into hydrophilic region; BA's methoxy group fits in hydrophilic region in ERα binding pocket
GN and BA docked in the LBD of **ERα**. Green regions represent hydrophobic regions; purple regions are hydrophilic.

5.2.4 **Combined techniques:**

Literature indicated that silymarin (milk thistle) and its major bioactive compound, silibinin, exhibited estrogenic activity *in vivo* (El-Shitany, Hegazy, and El-Desoky 2010) and in some *in vitro* assays (Dupuis et al. 2018). However, when these were screened for estrogenicity, the alkaline phosphatase assay indicated no significant estrogenic activity, but did suggest antiestrogenic activity (Figure 27).

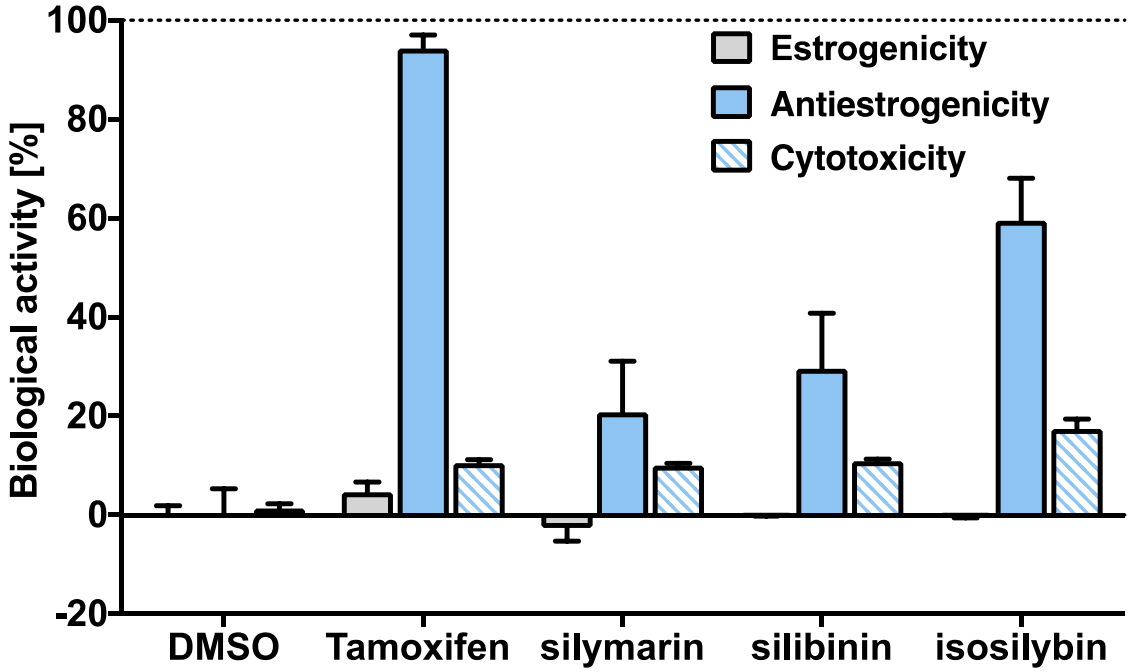

Figure 27: Evaluation of the (anti)estrogenic activity in the alkaline phosphatase induction assay.
Results are presented as reduction of 1 nM E_2 response. Tamoxifen (5 μM) was used as positive control for anti-estrogenic activity (94.3 % reduction). Cells were treated with 10 µM or 10 µg/ml of silymarin/silibinin/isosilybin. Results are the means ± SEM of at least three independent determinations in triplicate.

Silibinin is clearly too big fit the binding pocket, with the red colored part of the structure indicating portions of the compound that would be outside of the binding pocket. Moreover, even if entered the binding pocket, it does not interact with the important Glu. Thus, it would be unlikely to exhibit either strong estrogenic or antiestrogenic effects. This suggests that a metabolite of silibinin or another compound

may be responsible for the activity. (Figure 28). This project was not continued beyond this point at this time.

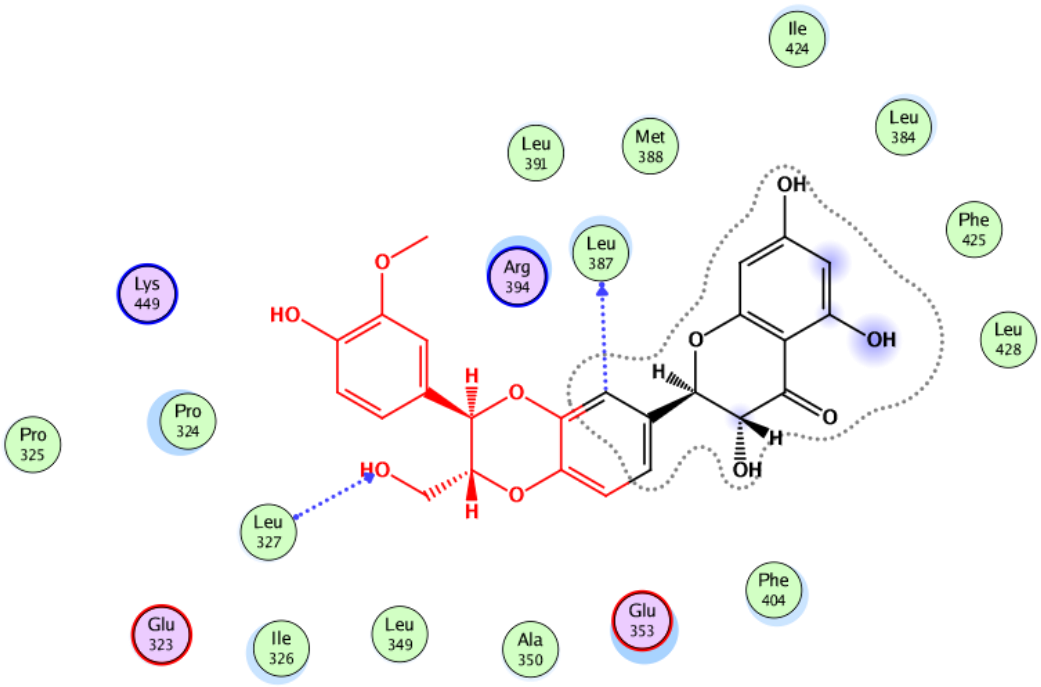

Figure 28:Silibinin too large for binding site of ERα.
2D representation of silibinin docked in the binding pocket of ERα. The black section falls within the binding pocket; the red portion falls outside of the binding pocket.

6 EFFECT OF PRESENCE OF ESTRADIOL ON ESTROGEN RECEPTOR ALPHA MEDIATED SUPPRESSION OF P450 1A1

6.1 Rationale

Previously, we demonstrated that estrogenic isoflavones (phytoestrogens) can suppress the *CYP1A1* expression in the detoxification pathway through ERα activation (chapter 3; Dunlap et al. 2017). However, this study was conducted without estrogens present. In premenopausal women, circulating estrogens are high. In postmenopausal women. The local production of estrogen in organs such as breast might not be that much compromised because estrogens, mainly estradiol (E_2) and estrone (E_1), are converted from testosterone and androstenedione by aromatase (P450 19A1) in adipose tissues in breast. To our knowledge a study of the role of estrogens on the modulation of oxidative estrogen metabolism by isoflavones has not been attempted.

To simplify the question of the effects of estrogens, this study was confined to only the epigenetic ERα mediated suppression of *CYP1A1*. In this study, a 4-day hydrolyzed soy (*Glycine max*) bean extract (SB) and its major bioactive isoflavones GN and DZ were chosen because of their strong estrogenic activity and lack of AhR mediated P450 1A1/1B1 induction (chapters 3 and 5). This extract was studied in ERα+ MCF-7 WS8 cells in three different *in vitro* model systems: no, low, and high estrogen. The effects within those systems are compared.

6.2 Results:

6.2.1 Estrogenic Compounds in Soy Bean extracts Selectively Suppress P450 1A1:

Upon treatment of MCF-7:WS8 cells with the phytoestrogens, GN or DZ (1 µM), or E_2 (1 nM), *CYP1A1*, but not *CYP1B1* was suppressed. Likewise, ER mediated P450 1A, but not AhR mediated P450 1A+1B1 induction/inhibition was selectively reduced in mammospheres treated with either 2 or 20 µM treatment.

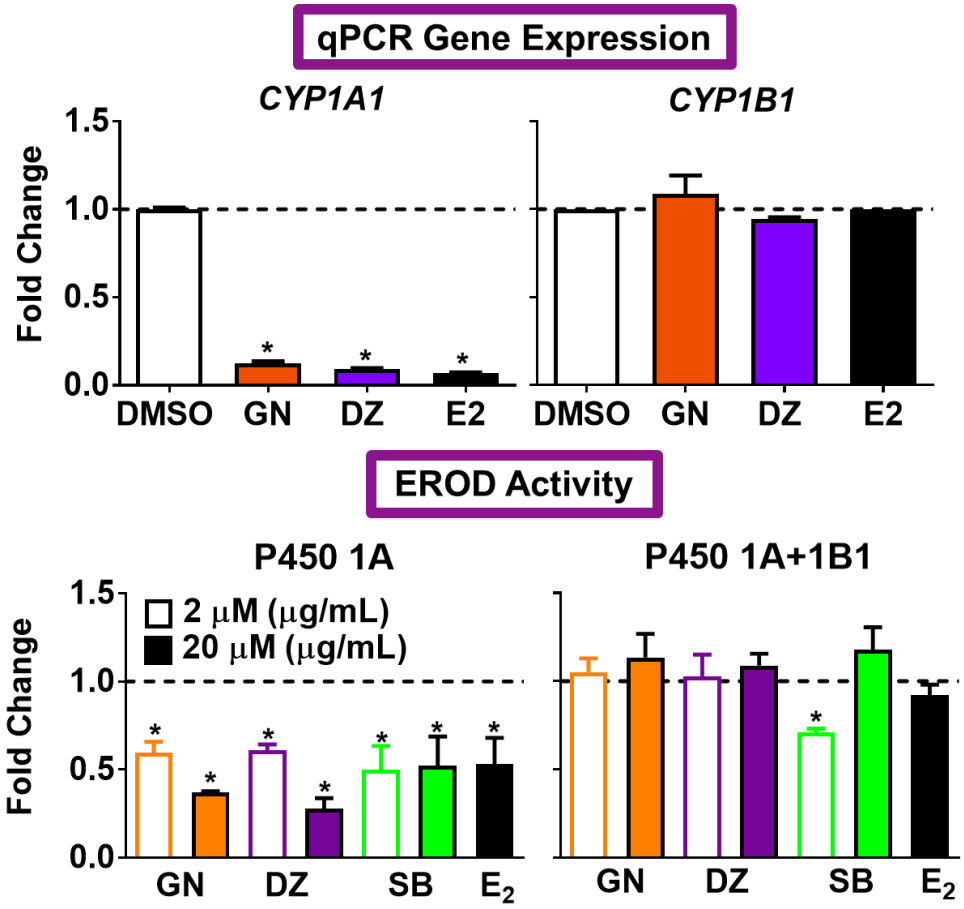

Figure 29: Estrogenic Compounds in Soy Bean Extract Selectively Suppress P450 1A1.

qPCR: *CYP1A1/1B1* gene expression of MCF-7 monolayer treated 24 hrs with GN, DZ (1 μM) or E_2 (1 nM) EROD activity of MCF-7 spheroids treated 24 hrs with GN, DZ (20 μM) or E_2 with or without TMS (1 μM). Normalized to DMSO. *p<0.05. Mean for TCDD (10 nM) AhR agonists treatment control (not shown): P450 1A: 14.54 fold change; P450 1A+ 1B1: 18.34 fold change. Normalized and T-test compared to DMSO+TMS (P450 1A) or DMSO (P450 1A+1B1) negative control. *p<0.05.

6.2.2 **ER mediated suppression of *CYP1A1* is dose dependent in the biologically relevant pM-nM range of E_2 treatment.**

Upon treatment of MCF-7:WS8 cells with E_2, *CYP1A1*, but not *CYP1B1* was suppressed dose dependently in the pM-nM range ($logIC_{50}$=-11.3 ± 0.2) determined by $logIC_{50}$s of three independent runs. Likewise, ER-mediated P450 1A, but not AhR mediated P450 1A+1B1 induction/inhibition was selectively inhibited dose responsively ($logIC_{50}$ =-10.3 ± 0.4) determined by determined by $logIC_{50}$s of three independent runs. This range is generally considered biologically relevant. A dose at the low end of the dose response (1 pM) was chosen for the “low estrogen” model system; a dose at the high end of the curve (1 nM) was chose for the “high estrogen” model.

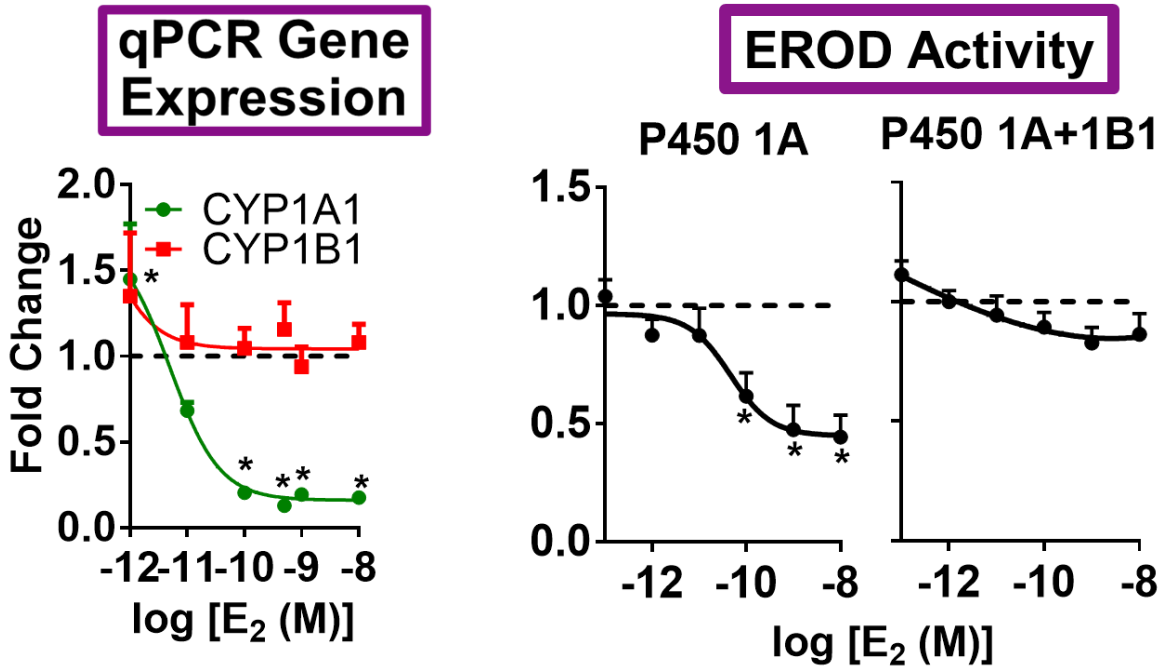

Figure 30 ER-mediated suppression of *CYP1A1* is dose dependent in the biologically relevant pM-nM range of E_2 treatment.
qPCR: *CYP1A1*/1B1 gene expression of MCF-7 monolayer treated with estradiol (E_2; 24 h.); EROD activity of MCF-7 spheroids treated with E_2 or ICI with or without 2,4,3′,5′-tetramethoxystilbene (TMS; 1 μM). Normalized to DMSO or DMSO+TMS (TMS; 1 μM). *p<0.05

6.2.3 **Cotreatment with E_2 for 24 h did not reverse *CYP1A1* selective downregulation (qPCR):**

In MCF-7/WS8 monolayer treated with phytoestrogen (1 μM) for 24 h., suppression of *CYP1A1*, but not *CYP1B1* occurred. Cells co-treated with E_2 do not change activity significantly differently than those only treated with a phytoestrogen.

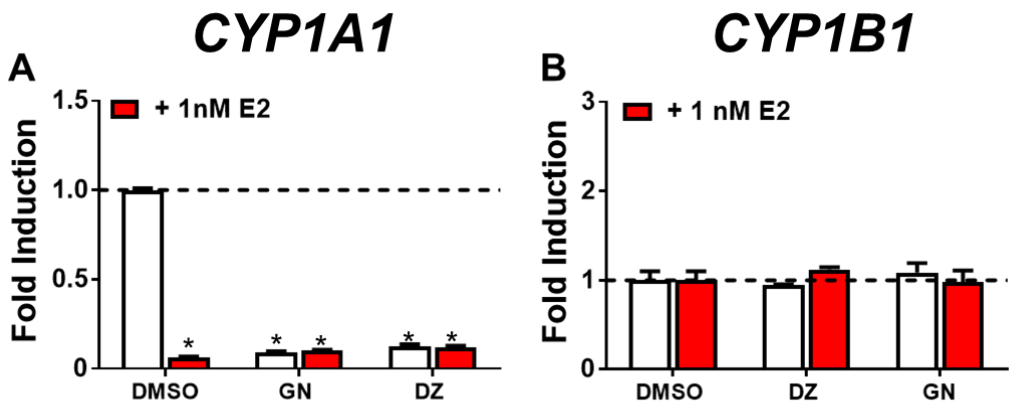

Figure 31: Cotreatment with E_2 for 24 h did not reverse *CYP1A1* selective downregulation.
qPCR analysis of ***CYP1A1*** and ***CYP1B1*** gene induction in MCF-7 cells treated with DMSO, GN, DZ, BA, or FN (1 µM) with or without estradiol (E_2;1 nM) for 24 hours. Normalized to and T-test compared to DMSO control. *p<0.05

6.2.4 **Pretreatment with E_2 Reverses ERα-mediated Suppression:**

Since women already have estrogen in their breast tissue when isoflavones are consumed, the effect of E_2 pretreatment on the P450 1A suppression was investigated. Spheroids were plated then pretreated with E_2, for 0, 1, 2, or 3 days before treatment for an additional 24 h with a fresh dose of same concentration of E_2, and EROD activity was determined. These results were normalized to cells only containing the negative control (DMSO; 0.1%). As expected from the previous three figures, the low dose of E_2 had no significant effect on ER-mediated P450 1A, or AhR mediated P450 1A+1B1 induction/inhibition while the high dose of E_2 (1 nM) spheres showed significant ER-

mediated P450 1A suppression, but not AhR mediated P450 1A+1B1 induction or inhibition. The P450 1A inhibition was reversed as pretreatment time increased, with 3 d pretreatment completely reversing the suppression. Thus, 3 d pretreatment was chosen for the model systems investigated.

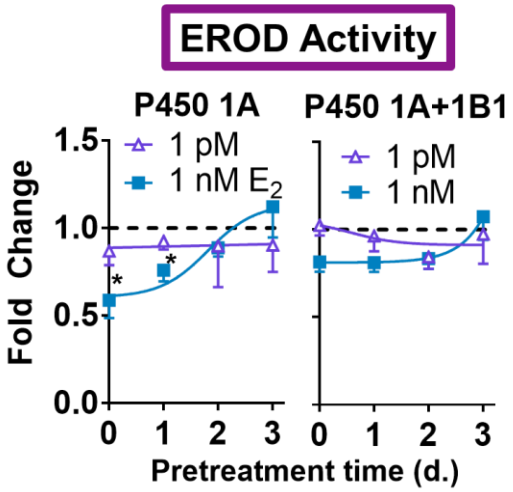

Figure 32: Pretreatment with E_2 Reverses ERα-mediated suppression.
EROD activity of MCF-7 spheroids treated were plated then pretreated with E_2, for 0, 1, 2, or 3 days before treatment for an additional 24 h with a fresh dose of same concentration of E_2, with (P450 1A) or without (P450 1A+1B1) TMS (1 μM) and EROD activity was read. Results were normalized to sphere diameter and DMSO of DMSO +TMS (1 μM). *$p<0.05$

6.2.5 **Pretreatment with E_2 Reverses P450 1A Selective Suppression:**

Mammospheres were pretreated with E_2 (1 pM or 1 nM) for 3 days then cotreated with fresh E_2 (1 pM or 1 nM, respectively) and extract or compound for 24 hours. With 1 pM E_2 pre/cotreatment, the suppression of P450 1A seen in Figure 29 begins to reverse, but suppression is still significant. With 1 nM E_2 pre/cotreatment, suppression is completely reversed, and induction occurred. (Figure 33)

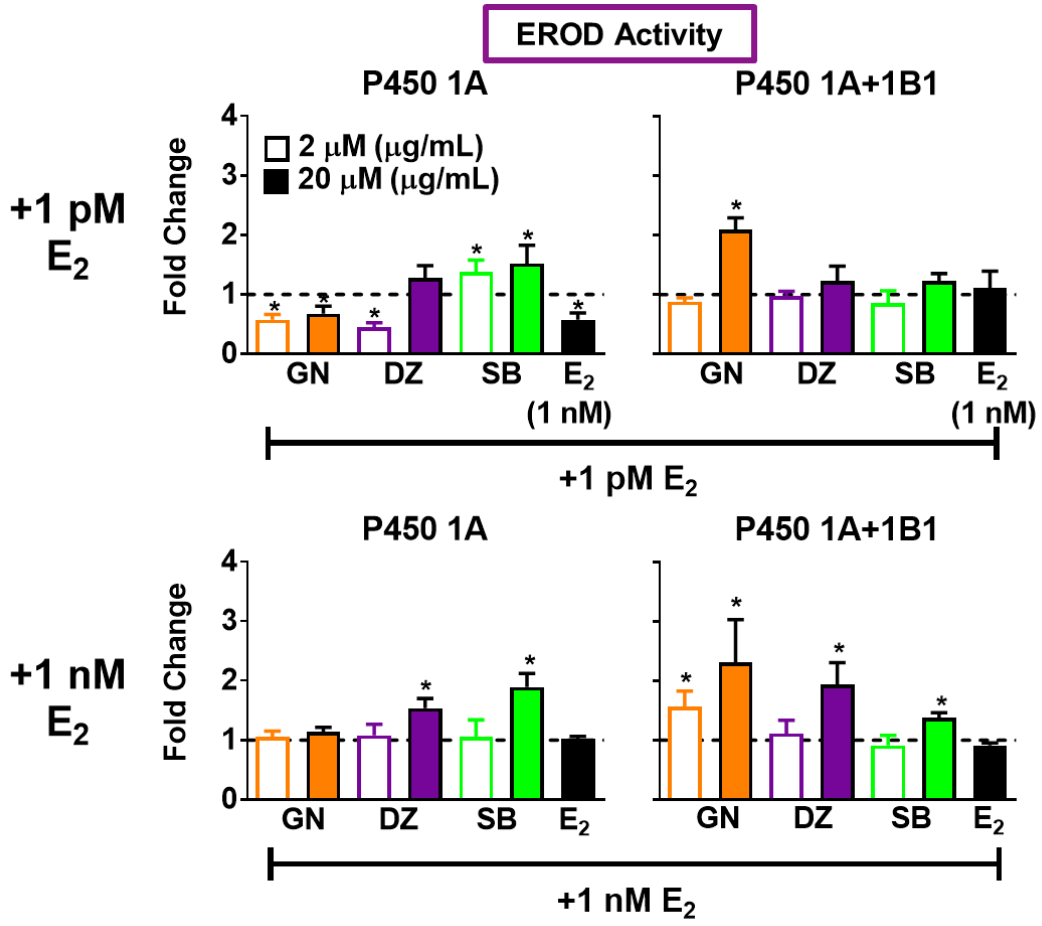

Figure 33: Pretreatment Reverses P450 1A Selective Suppression.
Mammospheres were pretreated with E_2 (1 pM or 1 nM) for 3 days then cotreated with fresh E_2 (1 pM or 1 nM, respectively) and extract or compound for 24 hours. Results were normalized to sphere diameter to respective E_2+DMSO control. Compared to respective E_2+DMSO control *$p<0.05$.

6.2.6 **AhR mediated induction of P450 1A1/1B1 not significantly affected by pretreatment with E_2:**

Since some isoflavones, such as biochanin A, act as AhR agonists (Chapter 3), the effect of E_2 pretreatment on AhR mediated induction was investigated. Cells in the no E_2, 1 pM E_2, and 1 nM E_2 model systems were treated with a strong AhR agonists, TCDD. No significant change occurred between models indicating that this difference in model system does not affect the AhR-mediated estrogen chemical carcinogenesis pathway (Figure 34).

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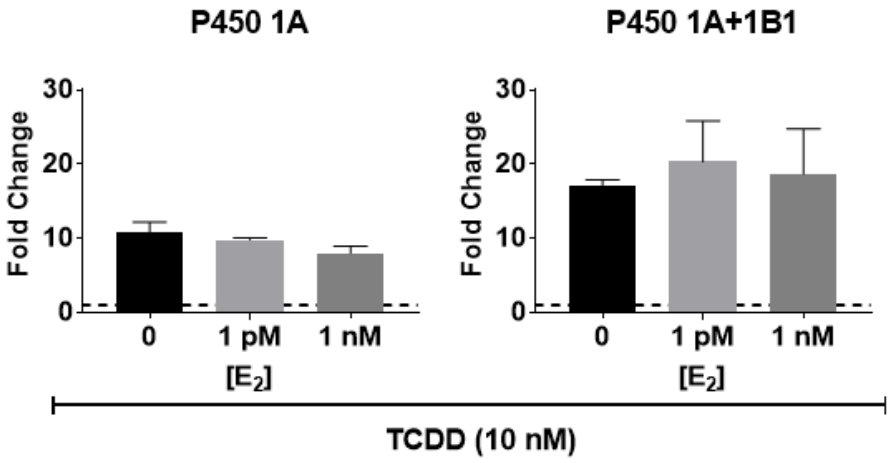

Figure 34: AhR mediated induction not significantly affected by pretreatment. Mammospheres were pretreated with E_2 (1 pM or 1 nM) for 3 days then cotreated with fresh E_2 (1 pM or 1 nM, respectively) and TCDD (10 nM) for 24 hours. Results were normalized to sphere diameter to respective DMSO control. *p<0.05

6.2.7 **Phytoestrogens do not selectively decrease 2-MeOE$_1$ metabolites of P450 1A1 mediated estrogen oxidative metabolism.**

To confirm our results, the 2-MeOE$_1$ and 4-MeOE$_1$ metabolites of cells treated with SB, GN, or DZ was measured. These metabolites also involve COMT enzymes (Dawling et al. 2001) (Figure 2), which can complicate the results.

To make sure to capture any possible estrogenic activity, cells were treated with a high concentration (20 µg/mL or µM) of extract or compound. GN significantly decreased both 2- and 4-MeOE$_1$ metabolites of P450 1A1 and P450 1B1 metabolism, respectively, and COMT metabolism, while DZ and SB did not significantly change metabolite levels.

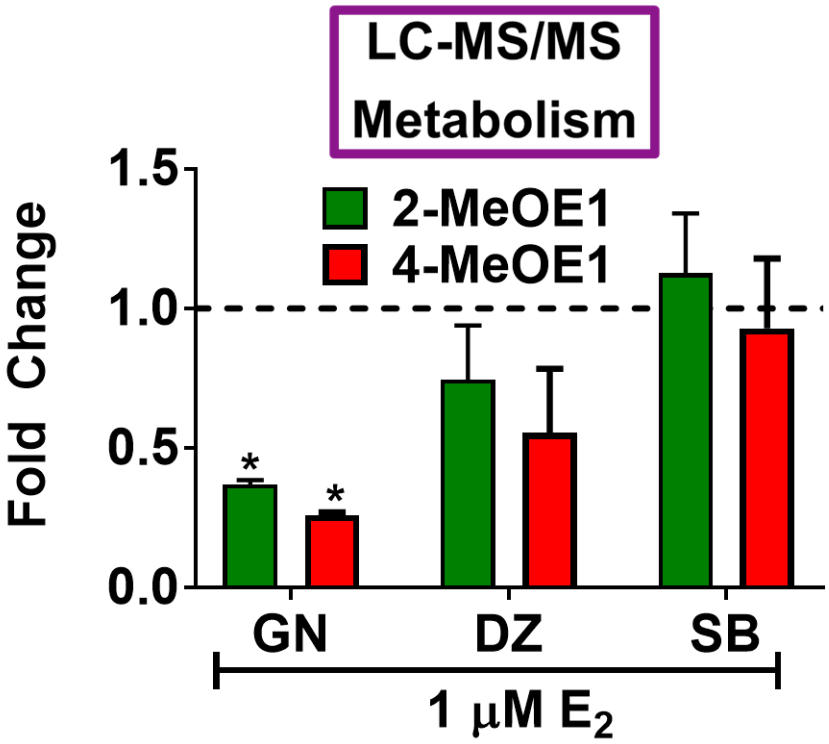

Figure 35: Phytoestrogens do not selectively decrease 2-MeOE_1 metabolites of P450 1A1 mediated estrogen oxidative metabolism.
GN lowers both 2- and 4-MeO**E_1** metabolites. LC-MS/MS measurement of monolayers pretreated with **E_2** (1 µM) for 3 days then cotreated with fresh **E_2** (1 µM) and extract or compound (20 µg/mL or µM) for 24 hours. Results were normalized to sphere diameter to respective **E_2**+DMSO control. Statistics compared to **E_2**+DMSO control. *p<0.05

6.3 Discussion:

Overall, our findings show that, in high E_2 containing systems, like women's breast tissue, P450 1A1 suppression by phytoestrogens should not. be a concern.
The mechanism behind the differences in modulation between the different model systems is not understood. We hypothesize that it may be related to a negative feedback loop leading to decreased ESR1 expression sometimes reported in MCF-7 cells (Ellison-Zelski, Solodin, and Alarid 2009), but this hypothesis still needs to be tested both in MCF-7 cells and in other ER+ cell lines.

6.3.1 Possible off target effects:

The induction of P450 enzymes by phytoestrogen treatment in E_2 containing model systems, can be explained by off target effects since ER is preferentially binding to E_2 not GN and DZ. GN and DZ are reported to be weak AhR agonists (Wall et al. 2012). The AhR mediated effect is an induction of both P450 1A and P450 1A+1B1 activity. The preferential induction of P450 1A by SB extract follows a similar trend as a known ERα antagonist, ICI 182,780 (Figure 13), indicating a possible ER antagonists activity. Further, GN is also a purported modulator of COMT (Lehmann, Jiang, and Wagner 2008), which may be why both metabolites decreased with GN treatment (Figure 35).

6.3.2 Isoflavone consumption and breast cancer risk in women:

Our findings are consistent with the conclusions of most recent studies finding a decreased or unchanged risk of cancer with increased isoflavone consumption both for cancer reoccurrence and initial cancer risk. A 2013 meta-analysis of cohort studies

found reduced risk of cancer reoccurrence in postmenopausal women consuming soy (Chi et al. 2013). Likewise, a systematic review including 131 articles: 40 randomized control trials, 11 uncontrolled trials, and 80 observational studies of red clover, isoflavones, or soy treatment found a lack of evidence of harm in relation to risk of breast cancer and other malignancies and possible preventive effects with doses common in Asian diets (Fritz et al. 2013). A 2017 analysis of data from the Breast Cancer Family Registry found that over a period of 113 months women who had been diagnosed with breast cancer and who were in the highest quartile of isoflavone intake had a 21% decrease in all-cause mortality compared to those in the lowest quartile. However, cancer risk involves many other pathways which can be affected by compounds in BDS and phytoestrogens are not recommended for cancer prevention, and caution is advised particularly at high doses (Camacho et al. 2016).

6.3.3 **Isoflavone consumption and 2 and 4-hydroxylation in women/animals:**

Several studies have investigated the effects of isoflavone rich diets on oxidative estrogen metabolism in women. Consistent with the findings of our study, studies in women have not found a selective decrease in urinary 2-hydroxylation metabolites of estrogens after isoflavone intake. A cross-over soy isoflavone feeding study measuring estrogens and their metabolites in urine of premenopausal women found that isoflavone consumption caused a decrease in estrogen levels, and a corresponding decrease in both 2- and 4- OHE_2 (Xu et al. 1998). In a similar study in premenopausal women, isoflavone rich diets increased the ratio of urinary 2/4-(OH) estrogens (Xu et al. 2000).

However, the results of these urinary studies may be confounded by estrogen metabolism outside of the breast tissue. A study of ovariectomized cynomolgus macaques fed an isoflavone depleted (soy-) or isoflavone rich (soy+) soy diet for 36 months found a slight increase in *CYP1A1* expression and slight decrease in *CYP1B1* expression in the mammary tissue in of the soy+ fed macaques (Scott et al. 2008). Moreover, decrease in both *CYP1A1 and CYP1B1* expression was found in the mammary tissues of rats treated for 4 days with estradiolbenzoate (EB), a prodrug for E_2 and compared rats treated with vehicle (Wang et al. 2018).

6.3.4 **Limitations:**

Although the activity of P450 1A1/1B1 is an important factor in estrogen oxidative metabolism, measuring it does not consider any effects occurring after P450 1A1/1B1 activity such modulation of COMT. Furthermore, measuring P450 1A1/1B1 activity cannot fully specify the cause of modulation of activity. For instance, although we know ICI preferentially increases P450 1A activity (Figure 13), we cannot say with certainty that the P450 1A induction with SB treatment in high E_2 containing models is due to an antiestrogenic effect without further testing. Likewise, we cannot say with certainty that an increase in P450 1A/1B1 activity is caused by an AhR agonist, only that an AhR agonists would have that effect. However, the suppression of P450 1A activity that is observed upon treatment with estrogenic compounds and reversed with ICI cotreatment (chapter 3) is reversed in models containing high levels of E_2.

The findings of our *in vitro* study bridge the gap between previous *in vitro* data and the above summarized *in vivo* and clinical studies, and further studies can be

conducted in similar models to yield more accurate data that can help women make informed choices about breast cancer chemoprevention.

7 CONCLUSIONS AND FUTURE DIRECTIONS

Women are increasingly using botanical dietary supplements to alleviate menopausal symptoms, but data regarding their safety and possible chemopreventive properties are unclear. In this study we took a novel approach to studying botanical extracts and their contained isoflavones in models of oxidative estrogen metabolism by P450 1A1 and P450 1B1 (estrogen chemical carcinogenesis). Their effects in no, low, and high E_2 systems were tested and differences between systems were noted.

RCE extract contains isoflavones acting as ER and AhR agonists. In estrogen-free systems, this produced an overall undesirable effect: At high concentrations, this extract showed preferential induction of the genotoxic P450 1B1 pathway, while at low concentrations, it selectively suppressed the P450 1A1 (detoxification) pathway. Both adverse effects were probably primarily due to the ER mediated suppression of *CYP1A1* as AhR mediated induction is not preferential for P450 1B1 in the absence of ER agonists. Thus, we decided to simplify the question and only consider the ER mediated effects of on oxidative estrogen metabolism.

However, to our knowledge, there was not an existing activity assay that robustly fits our needs. The 3D-EROD assay was created for this purpose and validated. Mammospheres (spheroids) were deliberately incorporated because of their increasing similarity to intracellular signaling in human breast cells. This assay was used to evaluate different botanicals including several soy bean extracts. From these extracts, we chose the most estrogenic, a four-day hydrolyzed extract, SB.

SB exerted a primarily estrogenic effect on oxidative estrogen metabolism. In an estrogen free model, it exerts the same adverse ER mediated effect seen in cells treated with low concentrations of RCE. However, in low estrogen (1 pM E_2) conditions, this trend began to reverse. In a high estrogen environment (1 nM E_2), this trend completely reversed. Since ER mediated suppression was reversed in cells pre- and cotreated with E_2.

Moreover, we have utilized the newly created screening assay and computational modeling to form hypotheses to answer questions regarding BDS for women's health and to better understand the binding sites of ERα and AhR. We also established that hydrolyzing a glycosylated extract can reveal biologically relevant activity and may be a helpful tool in future botanical studies.

Perhaps the biggest accomplishment in this study is the creation of what may be a more realistic *in vitro* model system to measure pathways involved in estrogen chemical carcinogenesis that is neither prohibitively expensive nor complicated. We employed 3D-mammospheres to form a more realistic intercellular environment, we investigated extracts that better reflect the bioactive compounds in the body rather than just using raw extracts, and we added estrogens into the study of pathways which involve the ER to better reflect the conditions present in women's breast tissue.

Less well-studied compounds and extracts *in vitro* and *in vivo* can be screened for effects on estrogen oxidative metabolism using this high-throughput model. Moreover, steps can be taken to create more realistic *in vitro* models that are easily

accessible to other scientists. For instance, when it is known that an endogenous ligand affects a pathway, it can be added to the assay and spheroid modeling can be utilized.

However, this study has limitations. the use of mammospheres may complicate our understanding of potential pathways due to increased complexity in these models. Moreover, although the activity of P450 1A1/1B1 is an important factor in estrogen oxidative metabolism, measuring it does not take into account any effects occurring after P450 1A1/1B1 activity such modulation of COMT. Further, measuring P450 1A1/1B1 activity cannot fully specify the cause of modulation of activity. For instance, although we know ICI preferentially increases P450 1A activity, we cannot say with certainty that the P450 1A induction with SB treatment in high E_2 containing models is due to an antiestrogenic effect without further testing. However, the suppression of P450 1A activity that is observed upon treatment with estrogenic compounds and reversed with ICI cotreatment is reversed in models containing high levels of E_2.

Moreover, the mechanism by which the estrogen mediated effects no longer occur with pretreatment should be investigated. Animal studies should include either transgenic mice that express human P450 1B1 or non-rodents and clinical data could be stratified to E_2 levels to further control for this factor.

Although, isoflavones in E_2 containing systems can exhibit off-target effects such as COMT downregulation and weak AhR agonists activity, overall, our findings show that, in high E_2 containing systems, like women's breast tissue, P450 1A1 suppression by phytoestrogens should not be a concern. These findings agree with the existing literature of clinical and *in vivo* studies in which the 2-hydroxylation pathway is not

selectively suppressed nor is the 4-OH pathway preferentially induced in either pre or post-menopausal woman or animals. Future mechanistic, animal, and clinical studies specifically addressing this question should be performed before results can be fully accepted. Future studies should include confirming the effects of the presence of E_2 on different systems along the estrogen oxidative metabolism pathway with experiments such as the XRE luciferase assay from chapter 3 and qPCR gene expression of *CYP1A1*/*CYP1B1*.

Although more work needs to be done, the findings of our *in vitro* study bridge the gap between previous *in vitro* data and the above summarized *in vivo* and clinical studies, and further studies can be conducted in similar models to yield more accurate data that can help women make informed choices about isoflavone consumption and breast cancer chemoprevention.

CITED WORKS

1997. "Breast cancer and hormone replacement therapy: collaborative reanalysis of data from 51 epidemiological studies of 52,705 women with breast cancer and 108,411 women without breast cancer. Collaborative Group on Hormonal Factors in Breast Cancer." *Lancet* 350 (9084):1047-59.

2002. "Breast cancer and breastfeeding: collaborative reanalysis of individual data from 47 epidemiological studies in 30 countries, including 50302 women with breast cancer and 96973 women without the disease." *Lancet* 360 (9328):187-95. doi: 10.1016/s0140-6736(02)09454-0.

Aidelsburger, P., S. Schauer, K. Grabein, and J. Wasem. 2012. "Alternative methods for the treatment of post-menopausal troubles." *GMS Health Technol. Assess.* doi: 10.3205/hta000101hta000101 [pii].

Al-Dhfyan, Abdullah, Ali Alhoshani, and Hesham M. Korashy. 2017. "Aryl hydrocarbon receptor/cytochrome P450 1A1 pathway mediates breast cancer stem cells expansion through PTEN inhibition and β-Catenin and Akt activation." *Molecular Cancer* 16 (1):14. doi: 10.1186/s12943-016-0570-y.

Amer, Dena AM, Georg Kretzschmar, Nicole Müller, Nicole Stanke, Dirk Lindemann, and Günter Vollmer. 2010. "Activation of transgenic estrogen receptor-beta by selected phytoestrogens in a stably transduced rat serotonergic cell line." *J. Steroid Biochem. Mol. Biol.* 120 (4):208-217.

Angus, William G. R., Michele C. Larsen, and Colin R. Jefcoate. 1999. "Expression of CYP1A1 and CYP1B1 depends on cell-specific factors in human breast cancer cell lines: role of estrogen receptor status." *Carcinogenesis* 20 (6):947-955. doi: 10.1093/carcin/20.6.947.

Atkinson, Charlotte, Ruth ML Warren, Evis Sala, Mitch Dowsett, Alison M Dunning, Catherine S Healey, Shirley Runswick, Nicholas E Day, and Sheila A Bingham. 2004. "Red clover-derived isoflavones and mammographic breast density: a double-blind, randomized, placebo-controlled trial [ISRCTN42940165]." *Breast Cancer Res.* 6 (3):170-179.

Bahia, H, JNE Ashman, L Cawkwell, M Lind, JRT Monson, PJ Drew, and J Greenman. 2002. "Karyotypic variation between independently cultured strains of the cell line MCF-7 identified by multicolour fluorescence in situ hybridization." *Int. J. Oncol.* 20 (3):489-494.

Balachander, G. M., S. A. Balaji, A. Rangarajan, and K. Chatterjee. 2015. "Enhanced Metastatic Potential in a 3D Tissue Scaffold toward a Comprehensive in Vitro Model for Breast Cancer Metastasis." *ACS Appl Mater Interfaces* 7 (50):27810-22. doi: 10.1021/acsami.5b09064.

Baumgart, Annette, Melanie Schmidt, Hans-Joachim Schmitz, and Dieter Schrenk. 2005. "Natural furocoumarins as inducers and inhibitors of cytochrome P450 1A1 in rat hepatocytes." *Biochemical Pharmacology* 69 (4):657-667. doi: https://doi.org/10.1016/j.bcp.2004.11.017.

Beatson, G. T. 1983. "On the treatment of inoperable cases of carcinoma of the mamma - suggestions for a new method of treatment, with illustrative cases." *Ca-a Cancer Journal for Clinicians* 33 (2):108-121. doi: 10.3322/canjclin.33.2.108.

Beischlag, T. V., and G. H. Perdew. 2005. "ER alpha-AHR-ARNT protein-protein interactions mediate estradiol-dependent transrepression of dioxin-inducible gene transcription." *J. Biol. Chem.* 280 (22):21607-11. doi: 10.1074/jbc.C500090200.

Bialesova, L., A. Novotna, D. Macejova, J. Brtko, and Z. Dvorak. 2015. "Agonistic effect of selected isoflavones on arylhydrocarbon receptor in a novel AZ-AhR transgenic gene reporter human cell line." *Gen. Physiol. Biophys.* 34 (3):331-4. doi: 10.4149/gpb_2015008.

Bolca, S., M. Urpi-Sarda, P. Blondeel, N. Roche, L. Vanhaecke, S. Possemiers, N. Al-Maharik, N. Botting, D. De Keukeleire, M. Bracke, A. Heyerick, C. Manach, and H. Depypere. 2010. "Disposition of soy isoflavones in normal human breast tissue." *Am J Clin Nutr* 91 (4):976-84. doi: 10.3945/ajcn.2009.28854.

Bolton, J. L. 2016. "Menopausal Hormone Therapy, Age, and Chronic Diseases: Perspectives on Statistical Trends." *Chem Res Toxicol* 29 (10):1583-1590. doi: 10.1021/acs.chemrestox.6b00272.

Bolton, Judy L, and Gregory RJ Thatcher. 2007. "Potential mechanisms of estrogen quinone carcinogenesis." *Chem. Res. Toxicol.* 21 (1):93-101.

Bolton, Judy L., and Tareisha L. Dunlap. 2017a. "Formation and biological targets of quinones: cytotoxic versus cytoprotective effects." *Chemical Research in Toxicology* 30 (1):13-37. doi: 10.1021/acs.chemrestox.6b00256.

Bolton, Judy L., and Tareisha L. Dunlap. 2017b. "Formation and biological targets of quinones: cytotoxic versus cytoprotective effects." *Chem. Res. Toxicol.* 30 (1):13-37. doi: 10.1021/acs.chemrestox.6b00256.

Booth, N. L., C. R. Overk, P. Yao, J. E. Burdette, D. Nikolic, S. N. Chen, J. L. Bolton, R. B. van Breemen, G. F. Pauli, and N. R. Farnsworth. 2006. "The chemical and biologic profile of a red clover (Trifolium pratense L.) phase II clinical extract." *J Altern Complement Med* 12 (2):133-9. doi: 10.1089/acm.2006.12.133.

Boucher, Beatrice A, Michelle Cotterchio, Laura N Anderson, Nancy Kreiger, Victoria A Kirsh, and Lilian U Thompson. 2013. "Use of isoflavone supplements is associated with reduced postmenopausal breast cancer risk." *Int. J. Cancer* 132 (6):1439-1450.

Bray, F., J. Ferlay, I. Soerjomataram, R. L. Siegel, L. A. Torre, and A. Jemal. 2018. "Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries." *CA Cancer J Clin* 68 (6):394-424. doi: 10.3322/caac.21492.

Brunelli, E., A. Minassi, G. Appendino, and L. Moro. 2007. "8-Prenylnaringenin, inhibits estrogen receptor-alpha mediated cell growth and induces apoptosis in MCF-7 breast cancer cells." *J Steroid Biochem Mol Biol* 107 (3-5):140-8. doi: 10.1016/j.jsbmb.2007.04.003.

Burdette, Joanna E, Jianghua Liu, Dan Lantvit, Eula Lim, Nancy Booth, Krishna PL Bhat, Samad Hedayat, Richard B Van Breemen, Andreas I Constantinou, and John M Pezzuto. 2002. "*Trifolium pratense* (red clover) exhibits estrogenic effects *in vivo* in ovariectomized Sprague-Dawley rats." *J. Nutr.* 132 (1):27-30.

Callero, Mariana A., and Andrea I. Loaiza-Pérez. 2011. "The role of aryl hydrocarbon receptor and crosstalk with estrogen receptor in response of breast cancer cells to the novel antitumor agents benzothiazoles and aminoflavone." *International journal of breast cancer* 2011:923250-923250. doi: 10.4061/2011/923250.

Camacho, P. M., S. M. Petak, N. Binkley, B. L. Clarke, S. T. Harris, D. L. Hurley, M. Kleerekoper, E. M. Lewiecki, P. D. Miller, H. S. Narula, R. Pessah-Pollack, V. Tangpricha, S. J. Wimalawansa, and N. B. Watts. 2016. "American Association Of Clinical Endocrinologists and American College of Endocrinology Clinical Practice Guidelines for The Diagnosis and Treatment of Postmenopausal Osteoporosis - 2016." *Endocr Pract* 22 (Suppl 4):1-42. doi: 10.4158/ep161435.Gl.

Cavalieri, E. L., and E. G. Rogan. 2016a. "Depurinating estrogen-DNA adducts, generators of cancer initiation: their minimization leads to cancer prevention." *Clin. Transl. Med.* 5 (1):12. doi: UNSP 1210.1186/s40169-016-0088-3.

Cavalieri, E., and E. Rogan. 2006. "Catechol quinones of estrogens in the initiation of breast, prostate, and other human cancers: keynote lecture." *Ann N Y Acad Sci* 1089:286-301. doi: 10.1196/annals.1386.042.

Cavalieri, Ercole L, and Eleanor G Rogan. 2016b. "Inhibition of depurinating estrogen-DNA adduct formation in the prevention of breast and other cancers." In *Trends in Breast Cancer Prevention*, edited by Jose Russo, 113-145. Springer International Publishing.

Chambert, T., D. A. Miller, and J. D. Nichols. 2015. "Modeling false positive detections in species occurrence data under different study designs." *Ecology* 96 (2):332-9. doi: 10.1890/14-1507.1.

Chan, H. Y., H. Wang, and L. K. Leung. 2003. "The red clover (*Trifolium pratense*) isoflavone biochanin A modulates the biotransformation pathways of 7,12-dimethylbenz[a]anthracene." *Br. J. Nutr.* 90 (1):87-92.

Chen, X. P., W. Li, X. F. Xiao, L. L. Zhang, and C. X. Liu. 2013. "Phytochemical and pharmacological studies on Radix Angelica sinensis." *Chin J Nat Med* 11 (6):577-87. doi: 10.1016/s1875-5364(13)60067-9.

Chi, F., R. Wu, Y. C. Zeng, R. Xing, Y. Liu, and Z. G. Xu. 2013. "Post-diagnosis soy food intake and breast cancer survival: a meta-analysis of cohort studies." *Asian Pac J Cancer Prev* 14 (4):2407-12. doi: 10.7314/apjcp.2013.14.4.2407.

Chiang, H. M., H. C. Chen, C. S. Wu, P. Y. Wu, and K. C. Wen. 2015. "Rhodiola plants: Chemistry and biological activity." *J Food Drug Anal* 23 (3):359-369. doi: 10.1016/j.jfda.2015.04.007.

Choi, S. Y., T. Y. Ha, J. Y. Ahn, S. R. Kim, K. S. Kang, I. K. Hwang, and S. Kim. 2008. "Estrogenic activities of isoflavones and flavones and their structure-activity relationships." *Planta Med.* 74 (1):25-32. doi: 10.1055/s-2007-993760.

Chun, Y. J., S. Kim, D. Kim, S. K. Lee, and F. P. Guengerich. 2001. "A new selective and potent inhibitor of human cytochrome P450 1B1 and its application to antimutagenesis." *Cancer Res* 61 (22):8164-70.

Cipriano, M., N. Freyer, F. Knospel, N. G. Oliveira, R. Barcia, P. E. Cruz, H. Cruz, M. Castro, J. M. Santos, K. Zeilinger, and J. P. Miranda. 2017. "Self-assembled 3D spheroids and hollow-fibre bioreactors improve MSC-derived hepatocyte-like cell maturation in vitro." *Archives of Toxicology* 91 (4):1815-1832. doi: 10.1007/s00204-016-1838-0.

Correia, Ana Luísa, and Mina J Bissell. 2012. "The tumor microenvironment is a dominant force in multidrug resistance." *Drug resistance updates* 15 (1-2):39-49.

Dallal, C. M., J. A. Tice, D. S. Buist, D. C. Bauer, J. V. Lacey, Jr., J. A. Cauley, T. F. Hue, A. Lacroix, R. T. Falk, R. M. Pfeiffer, B. J. Fuhrman, T. D. Veenstra, X. Xu,

and L. A. Brinton. 2014. "Estrogen metabolism and breast cancer risk among postmenopausal women: a case-cohort study within B~FIT." *Carcinogenesis* 35 (2):346-55. doi: 10.1093/carcin/bgt367.

Dawling, S., N. Roodi, R. L. Mernaugh, X. Wang, and F. F. Parl. 2001. "Catechol-O-methyltransferase (COMT)-mediated metabolism of catechol estrogens: comparison of wild-type and variant COMT isoforms." *Cancer Res* 61 (18):6716-22.

de Lemos, Mário L. 2001. "Effects of soy phytoestrogens genistein and daidzein on breast cancer growth." *Ann. Pharmacother.* 35 (9):1118-1121.

De Witt Hamer, P. C., A. A. G. Van Tilborg, P. P. Eijk, P. Sminia, D. Troost, C. J. F. Van Noorden, B. Ylstra, and S. Leenstra. 2007. "The genomic profile of human malignant glioma is altered early in primary cell culture and preserved in spheroids." *Oncogene* 27:2091. doi: 10.1038/sj.onc.1210850

Dean W. Roberts, Daniel R. Doerge, Mona I. Churchwell, Goncalo Gamboa da Costa, M. Matilde Marques, William H. Tolleson. 2004. "Inhibition of extrahepatic human cytochromes P450 1A1 and 1B1 by metabolism of isoflavones found in Trifolium pratense (red clover)." *J. Agric. Food Chem.* 52:6623-6632.

Dietz, B. M., A. Hajirahimkhan, T. L. Dunlap, and J. L. Bolton. 2016. "Botanicals and their bioactive phytochemicals for women's health." *Pharmacol. Rev.* 68 (4):1026-1073. doi: 10.1124/pr.115.010843.

Dong, J., Q. Zhang, Q. Cui, G. Huang, X. Pan, and S. Li. 2016. "Flavonoids and naphthoflavonoids: wider roles in the modulation of cytochrome P450 family 1 enzymes." *ChemMedChem* 11 (19):2102-2118. doi: 10.1002/cmdc.201600316.

Dong, Y., C. Stephens, C. Walpole, J. E. Swedberg, G. M. Boyle, P. G. Parsons, M. A. McGuckin, J. M. Harris, and J. A. Clements. 2013. "Paclitaxel resistance and multicellular spheroid formation are induced by kallikrein-related peptidase 4 in serous ovarian cancer cells in an ascites mimicking microenvironment." *PLoS One* 8 (2):e57056. doi: 10.1371/journal.pone.0057056.

Dowsett, M., J. F. Forbes, R. Bradley, J. Ingle, T. Aihara, J. Bliss, F. Boccardo, A. Coates, R. C. Coombes, J. Cuzick, P. Dubsky, M. Gnant, M. Kaufmann, L. Kilburn, F. Perrone, D. Rea, B. Thurlimann, C. van de Velde, H. Pan, R. Peto, C. Davies, R. Gray, M. Baum, A. Buzdar, J. Cuzick, M. Dowsett, J. F. Forbes, I. Sestak, C. Markopoulos, P. Dubsky, C. Fesl, M. Gnant, R. Jakesz, A. Coates, M. Colleoni, J. F. Forbes, R. Gelber, M. Regan, M. Kaufmann, G. von Minckwitz, J. Bliss, A. Coates, R. C. Coombes, J. F. Forbes, L. Kilburn, C. Snowdon, F. Boccardo, F. Perrone, P. Goss, J. Ingle, K. Pritchard, S. Anderson, J. Costantino, E. Mamounas, T. Aihara, Y. Ohashi, T. Watanabe, E. Bastiaannet, C. van de Velde, D. Rea, and Trialists Early Breast Canc. 2015. "Aromatase inhibitors versus tamoxifen in early breast cancer: patient-level meta-analysis of the randomised trials." *Lancet* 386 (10001):1341-1352. doi: 10.1016/s0140-6736(15)61074-1.

Dunlap, T. L., S. Wang, C. Simmler, S. N. Chen, G. F. Pauli, B. M. Dietz, and J. L. Bolton. 2015a. "Differential Effects of Glycyrrhiza Species on Genotoxic Estrogen Metabolism: Licochalcone A Downregulates P450 1B1, whereas Isoliquiritigenin Stimulates It." *Chem Res Toxicol* 28 (8):1584-94. doi: 10.1021/acs.chemrestox.5b00157.

Dunlap, Tareisha L, Shuai Wang, Charlotte Simmler, Shao-Nong Chen, Guido F Pauli, Birgit M Dietz, and Judy L Bolton. 2015b. "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.

Dunlap, Tareisha L., Caitlin E. Howell, Nita Mukand, Shao-Nong Chen, Guido F. Pauli, Birgit M. Dietz, and Judy L. Bolton. 2017. "Red Clover Aryl Hydrocarbon Receptor (AhR) and Estrogen Receptor (ER) Agonists Enhance Genotoxic Estrogen Metabolism." *Chemical Research in Toxicology* 30 (11):2084-2092. doi: 10.1021/acs.chemrestox.7b00237.

Dupuis, M. L., F. Conti, A. Maselli, M. T. Pagano, A. Ruggieri, S. Anticoli, A. Fragale, L. Gabriele, M. C. Gagliardi, M. Sanchez, F. Ceccarelli, C. Alessandri, G. Valesini, E. Ortona, and M. Pierdominici. 2018. "The Natural Agonist of Estrogen Receptor beta Silibinin Plays an Immunosuppressive Role Representing a Potential Therapeutic Tool in Rheumatoid Arthritis." *Front Immunol* 9:1903. doi: 10.3389/fimmu.2018.01903.

Einem Lindeman, T., M. C. Poirier, and R. L. Divi. 2011. "The resveratrol analogue, 2,3',4,5'-tetramethoxystilbene, does not inhibit CYP gene expression, enzyme activity and benzo[a]pyrene-DNA adduct formation in MCF-7 cells exposed to benzo[a]pyrene." *Mutagenesis* 26 (5):629-35. doi: 10.1093/mutage/ger024.

El-Shitany, N. A., S. Hegazy, and K. El-Desoky. 2010. "Evidences for antiosteoporotic and selective estrogen receptor modulator activity of silymarin compared with ethinylestradiol in ovariectomized rats." *Phytomedicine* 17 (2):116-25. doi: 10.1016/j.phymed.2009.05.012.

Elameen, A., S. Dragland, and S. S. Klemsdal. 2010. "Bioactive compounds produced by clones of Rhodiola rosea maintained in the Norwegian germplasm collection." *Pharmazie* 65 (8):618-23.

Eliassen, A. H., S. A. Missmer, S. S. Tworoger, and S. E. Hankinson. 2008. "Circulating 2-hydroxy- and 16alpha-hydroxy estrone levels and risk of breast cancer among postmenopausal women." *Cancer Epidemiol Biomarkers Prev* 17 (8):2029-35. doi: 10.1158/1055-9965.Epi-08-0262.

Ellison-Zelski, Stephanie J., Natalia M. Solodin, and Elaine T. Alarid. 2009. "Repression of ESR1 through Actions of Estrogen Receptor Alpha and Sin3A at the Proximal Promoter." *Molecular and Cellular Biology* 29 (18):4949-4958. doi: 10.1128/mcb.00383-09.

Falk, R. T., L. A. Brinton, J. F. Dorgan, B. J. Fuhrman, T. D. Veenstra, X. Xu, and G. L. Gierach. 2013. "Relationship of serum estrogens and estrogen metabolites to postmenopausal breast cancer risk: a nested case-control study." *Breast Cancer Res* 15 (2):R34. doi: 10.1186/bcr3416.

Fang, H., W. Tong, L. M. Shi, R. Blair, R. Perkins, W. Branham, B. S. Hass, Q. Xie, S. L. Dial, C. L. Moland, and D. M. Sheehan. 2001. "Structure-activity relationships for a large diverse set of natural, synthetic, and environmental estrogens." *Chem Res Toxicol* 14 (3):280-94. doi: 10.1021/tx000208y.

Forli, Stefano, Ruth Huey, Michael E. Pique, Michel F. Sanner, David S. Goodsell, and Arthur J. Olson. 2016. "Computational protein-ligand docking and virtual drug

screening with the AutoDock suite." *Nature protocols* 11 (5):905-919. doi: 10.1038/nprot.2016.051.

Franco, O. H., R. Chowdhury, J. Troup, T. Voortman, S. Kunutsor, M. Kavousi, C. Oliver-Williams, and T. Muka. 2016. "Use of plant-based therapies and menopausal symptoms A systematic review and meta-analysis." *J. Am. Med. Assoc.* 315 (23):2554-2563. doi: 10.1001/jama.2016.8012.

Friedrich, Juergen, Claudia Seidel, Reinhard Ebner, and Leoni A Kunz-Schughart. 2009. "Spheroid-based drug screen: considerations and practical approach." *Nature protocols* 4 (3):309.

Fritz, H., D. Seely, G. Flower, B. Skidmore, R. Fernandes, S. Vadeboncoeur, D. Kennedy, K. Cooley, R. Wong, S. Sagar, E. Sabri, and D. Fergusson. 2013. "Soy, red clover, and isoflavones and breast cancer: a systematic review." *PLoS One* 8 (11):e81968. doi: 10.1371/journal.pone.0081968.

Fuhrman, B. J., C. Schairer, M. H. Gail, J. Boyd-Morin, X. Xu, L. Y. Sue, S. S. Buys, C. Isaacs, L. K. Keefer, T. D. Veenstra, C. D. Berg, R. N. Hoover, and R. G. Ziegler. 2012. "Estrogen metabolism and risk of breast cancer in postmenopausal women." *J Natl Cancer Inst* 104 (4):326-39. doi: 10.1093/jnci/djr531.

Gaskell, Harriet, Parveen Sharma, Helen E. Colley, Craig Murdoch, Dominic P. Williams, and Steven D. Webb. 2016. "Characterization of a functional C3A liver spheroid model." *Toxicology Research* 5 (4):1053-1065. doi: 10.1039/C6TX00101G.

Geller, S. E., L. P. Shulman, R. B. van Breemen, S. Banuvar, Y. Zhou, G. Epstein, S. Hedayat, D. Nikolic, E. C. Krause, C. E. Piersen, J. L. Bolton, G. F. Pauli, and N. R. Farnsworth. 2009. "Safety and efficacy of black cohosh and red clover for the management of vasomotor symptoms: a randomized controlled trial." *Menopause* 16 (6):1156-66. doi: 10.1097/gme.0b013e3181ace49b.

Geller, S. E., and L. Studee. 2005. "Botanical and dietary supplements for menopausal symptoms: what works, what does not." *J. Womens Health* 14 (7):634-49. doi: 10.1089/jwh.2005.14.634.

Gerbarg, P. L., and R. P. Brown. 2016. "Pause menopause with Rhodiola rosea, a natural selective estrogen receptor modulator." *Phytomedicine* 23 (7):763-9. doi: 10.1016/j.phymed.2015.11.013.

Gertsch, J. 2011. "Botanical drugs, synergy, and network pharmacology: forth and back to intelligent mixtures." *Planta Med* 77 (11):1086-98. doi: 10.1055/s-0030-1270904.

Gong, Ping, Zeynep Madak-Erdogan, Jodi A Flaws, David J Shapiro, John A Katzenellenbogen, and Benita S Katzenellenbogen. 2016. "Estrogen receptor-α and aryl hydrocarbon receptor involvement in the actions of botanical estrogens in target cells." *Mol. Cell. Endocrinol.* 437:190-200.

Gupta, Mona, Andrew McDougal, and Stephen Safe. 1998. "Estrogenic and antiestrogenic activities of 16α-and 2-hydroxy metabolites of 17β-estradiol in MCF-7 and T47D human breast cancer cells." *J. Steroid Biochem. Mol. Biol.* 67 (5):413-419.

Hajirahimkhan, A., B. M. Dietz, and J. L. Bolton. 2013a. "Botanical modulation of menopausal symptoms: mechanisms of action?" *Planta Med.* 79 (7):538-53. doi: 10.1055/s-0032-1328187.

Hajirahimkhan, Atieh, Birgit M. Dietz, and Judy L. Bolton. 2013b. "Botanical modulation of menopausal symptoms: mechanisms of action?" *Planta medica* 79 (7):538-553. doi: 10.1055/s-0032-1328187.

Hajirahimkhan, Atieh, Charlotte Simmler, Yang Yuan, Jeffrey R. Anderson, Shao-Nong Chen, Dejan Nikolić, Birgit M. Dietz, Guido F. Pauli, Richard B. van Breemen, and Judy L. Bolton. 2013. "Evaluation of Estrogenic Activity of Licorice Species in Comparison with Hops Used in Botanicals for Menopausal Symptoms." *PLoS ONE* 8 (7):e67947. doi: 10.1371/journal.pone.0067947.

Han, E. H., J. Y. Kim, and H. G. Jeong. 2006a. "Effect of biochanin A on the aryl hydrocarbon receptor and cytochrome P450 1A1 in MCF-7 human breast carcinoma cells." *Arch. Pharm. Res.* 29 (7):570-6.

Han, Eun-Hee, Tae-Cheon Jeong, and Hye-Gwang Jeong. 2007. "Effects of formononetin on the aryl hydrocarbon receptor and 7,12-dimethylbenz [a] anthracene-induced cytochrome P450 1A1 in MCF-7 human breast carcinoma cells." *Toxicol. Res.* 23 (2):135-142.

Han, Eun Hee, Ji Young Kim, and Hye Gwang Jeong. 2006b. "Effect of biochanin A on the aryl hydrocarbon receptor and cytochrome P450 1A1 in MCF-7 human breast carcinoma cells." *Archives of pharmacal research* 29 (7):570-576.

Haycock, J. W. 2011. "3D cell culture: a review of current approaches and techniques." *Methods Mol Biol* 695:1-15. doi: 10.1007/978-1-60761-984-0_1.

He, Fen-Jin, and Jin-Qiang Chen. 2013. "Consumption of soybean, soy foods, soy isoflavones and breast cancer incidence: Differences between Chinese women and women in Western countries and possible mechanisms." *Food Science and Human Wellness* 2 (3):146-161. doi: https://doi.org/10.1016/j.fshw.2013.08.002.

Helguero, L. A., M. H. Faulds, J. A. Gustafsson, and L. A. Haldosen. 2005. "Estrogen receptors alfa (ERalpha) and beta (ERbeta) differentially regulate proliferation and apoptosis of the normal murine mammary epithelial cell line HC11." *Oncogene* 24 (44):6605-16. doi: 10.1038/sj.onc.1208807.

Hemachandra, LP, P Madhubhani, R Chandrasena, P Esala, S. N. Chen, Matthew Main, David C Lankin, Robert A Scism, Birgit M Dietz, Guido F Pauli, G. R. Thatcher, and J. L. Bolton. 2012. "Hops (*Humulus lupulus*) inhibits oxidative estrogen metabolism and estrogen-induced malignant transformation in human mammary epithelial cells (MCF-10A)." *Cancer Prev. Res.* 5 (1):73-81.

Hsieh, Ching-Yi, Ross C Santell, Sandra Z Haslam, and William G Helferich. 1998. "Estrogenic effects of genistein on the growth of estrogen receptor-positive human breast cancer (MCF-7) cells *in vitro* and *in vivo*." *Cancer Res.* 58 (17):3833-3838.

Huang, H. J., P. H. Chiang, and S. H. Chen. 2011. "Quantitative analysis of estrogens and estrogen metabolites in endogenous MCF-7 breast cancer cells by liquid chromatography-tandem mass spectrometry." *Journal of Chromatography B-*

Analytical Technologies in the Biomedical and Life Sciences 879 (20):1748-1756. doi: 10.1016/j.jchromb.2011.04.020.

Hunter, D. J., D. Spiegelman, H. O. Adami, P. A. van den Brandt, A. R. Folsom, R. A. Goldbohm, S. Graham, G. R. Howe, L. H. Kushi, J. R. Marshall, A. B. Miller, F. E. Speizer, W. Willett, A. Wolk, and S. S. Yaun. 1997. "Non-dietary factors as risk factors for breast cancer, and as effect modifiers of the association of fat intake and risk of breast cancer." *Cancer Causes Control* 8 (1):49-56.

Jiang, S. Y., D. M. Wolf, J. M. Yingling, C. Chang, and V. C. Jordan. 1992. "An estrogen-receptor positive MCF-7 Clone that is resistant to antiestrogens and estradiol." *Mol. Cell. Endocrinol.* 90 (1):77-86. doi: Doi 10.1016/0303-7207(92)90104-E.

Jin, U. H., Y. Cheng, H. Park, L. A. Davidson, E. S. Callaway, R. S. Chapkin, A. Jayaraman, A. Asante, C. Allred, E. A. Weaver, and S. Safe. 2017. "Short Chain Fatty Acids Enhance Aryl Hydrocarbon (Ah) Responsiveness in Mouse Colonocytes and Caco-2 Human Colon Cancer Cells." *Sci Rep* 7 (1):10163. doi: 10.1038/s41598-017-10824-x.

Kim, S. H., E. C. Henry, D. K. Kim, Y. H. Kim, K. J. Shin, M. S. Han, T. G. Lee, J. K. Kang, T. A. Gasiewicz, S. H. Ryu, and P. G. Suh. 2006. "Novel compound 2-methyl-2H-pyrazole-3-carboxylic acid (2-methyl-4-o-tolylazo-phenyl)-amide (CH-223191) prevents 2,3,7,8-TCDD-induced toxicity by antagonizing the aryl hydrocarbon receptor." *Mol Pharmacol* 69 (6):1871-8. doi: 10.1124/mol.105.021832.

Kleiner, Heather E., Melissa J. Reed, and John DiGiovanni. 2003. "Naturally Occurring Coumarins Inhibit Human Cytochromes P450 and Block Benzo[a]pyrene and 7,12-Dimethylbenz[a]anthracene DNA Adduct Formation in MCF-7 Cells." *Chemical Research in Toxicology* 16 (3):415-422. doi: 10.1021/tx025636d.

Kligman, L, and J Younus. 2010. "Management of hot flashes in women with breast cancer." *Curr. Oncol.* 17 (1):81.

Kolodziejczyk-Czepas, J. 2012. "Trifolium species-derived substances and extracts-biological activity and prospects for medicinal applications." *J. Ethnopharmacol.* 143 (1):14-23. doi: 10.1016/j.jep.2012.06.048.

Lai, K. P., M. H. Wong, and Chris K. C. Wong. 2004. "Modulation of AhR-Mediated CYP1A1 mRNA and EROD Activities by 17β-Estradiol and Dexamethasone in TCDD-Induced H411E Cells." *Toxicological Sciences* 78 (1):41-49. doi: 10.1093/toxsci/kfh045.

Lehmann, L., L. Jiang, and J. Wagner. 2008. "Soy isoflavones decrease the catechol-O-methyltransferase-mediated inactivation of 4-hydroxyestradiol in cultured MCF-7 cells." *Carcinogenesis* 29 (2):363-370. doi: 10.1093/carcin/bgm235.

Lenfant, F., F. Tremollieres, P. Gourdy, and J. F. Arnal. 2011. "Timing of the vascular actions of estrogens in experimental and human studies: why protective early, and not when delayed?" *Maturitas* 68 (2):165-73. doi: 10.1016/j.maturitas.2010.11.016.

Leung, Hau Y, Lai Hang Yung, Ching Ho Poon, Guoli Shi, A-Lien Lu, and Lai K Leung. 2008. "Genistein protects against polycyclic aromatic hydrocarbon-induced oxidative DNA damage in non-cancerous breast cells MCF-10A." *Br. J. Nutr.* 101 (2):257-262.

Li, Y., K. Wang, Y. Z. Jiang, X. W. Chang, C. F. Dai, and J. Zheng. 2014. "2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) inhibits human ovarian cancer cell proliferation." *Cell Oncol (Dordr)* 37 (6):429-37. doi: 10.1007/s13402-014-0206-4.

Lin, R. Z., and H. Y. Chang. 2008. "Recent advances in three-dimensional multicellular spheroid culture for biomedical research." *Biotechnol J* 3 (9-10):1172-84. doi: 10.1002/biot.200700228.

Liu, H., J. Wang, W. Zhou, Y. Wang, and L. Yang. 2013. "Systems approaches and polypharmacology for drug discovery from herbal medicines: an example using licorice." *J Ethnopharmacol* 146 (3):773-93. doi: 10.1016/j.jep.2013.02.004.

Lu, Fang, Muhammad Zahid, Cheng Wang, Muhammad Saeed, Ercole L. Cavalieri, and Eleanor G. Rogan. 2008. "Resveratrol Prevents Estrogen-DNA Adduct Formation and Neoplastic Transformation in MCF-10F Cells." *Cancer Prevention Research* 1 (2):135-145. doi: 10.1158/1940-6207.Capr-08-0037.

Maguire, S. L., B. Peck, P. T. Wai, J. Campbell, H. Barker, A. Gulati, F. Daley, S. Vyse, P. Huang, C. J. Lord, G. Farnie, K. Brennan, and R. Natrajan. 2016. "Three-dimensional modelling identifies novel genetic dependencies associated with breast cancer progression in the isogenic MCF10 model." *J Pathol* 240 (3):315-328. doi: 10.1002/path.4778.

Manson, J. E., R. T. Chlebowski, M. L. Stefanick, A. K. Aragaki, J. E. Rossouw, R. L. Prentice, G. Anderson, B. V. Howard, C. A. Thomson, A. Z. LaCroix, J. Wactawski-Wende, R. D. Jackson, M. Limacher, K. L. Margolis, S. Wassertheil-Smoller, S. A. Beresford, J. A. Cauley, C. B. Eaton, M. Gass, J. Hsia, K. C. Johnson, C. Kooperberg, L. H. Kuller, C. E. Lewis, S. Liu, L. W. Martin, J. K. Ockene, M. J. O'Sullivan, L. H. Powell, M. S. Simon, L. Van Horn, M. Z. Vitolins, and R. B. Wallace. 2013. "Menopausal hormone therapy and health outcomes during the intervention and extended poststopping phases of the Women's Health Initiative randomized trials." *Jama* 310 (13):1353-68. doi: 10.1001/jama.2013.278040.

Marques, M., L. Laflamme, and L. Gaudreau. 2013. "Estrogen receptor alpha can selectively repress dioxin receptor-mediated gene expression by targeting DNA methylation." *Nucleic Acids Res* 41 (17):8094-106. doi: 10.1093/nar/gkt595.

Maximov, Philipp Y., Cynthia B. Myers, Ramona F. Curpan, Joan S. Lewis-Wambi, and V. Craig Jordan. 2010. "Structure-function relationships of estrogenic triphenylethylenes related to endoxifen and 4-hydroxytamoxifen." *Journal of medicinal chemistry* 53 (8):3273-3283. doi: 10.1021/jm901907u.

Medjakovic, S, and A Jungbauer. 2008. "Red clover isoflavones biochanin A and formononetin are potent ligands of the human aryl hydrocarbon receptor." *J. Steroid Biochem. Mol. Biol.* 108 (1):171-177.

Mehta, Geeta, Amy Y Hsiao, Marylou Ingram, Gary D Luker, and Shuichi Takayama. 2012. "Opportunities and challenges for use of tumor spheroids as models to test drug delivery and efficacy." *Journal of Controlled Release* 164 (2):192-204.

Minino, A. M. 2011. "Death in the United States, 2009." *NCHS Data Brief* (64):1-8.

Mintziori, G., I. Lambrinoudaki, D. G. Goulis, I. Ceausu, H. Depypere, C. T. Erel, F. R. Perez-Lopez, K. Schenck-Gustafsson, T. Simoncini, F. Tremollieres, and M. Rees. 2015. "EMAS position statement: Non-hormonal management of menopausal vasomotor symptoms." *Maturitas* 81 (3):410-3. doi: 10.1016/j.maturitas.2015.04.009.

Moore, Steven C., Charles E. Matthews, Xiao Ou Shu, Kai Yu, Mitchell H. Gail, Xia Xu, Bu-Tian Ji, Wong-Ho Chow, Qiuyin Cai, Honglan Li, Gong Yang, David Ruggieri, Jennifer Boyd-Morin, Nathaniel Rothman, Robert N. Hoover, Yu-Tang Gao, Wei Zheng, and Regina G. Ziegler. 2016. "Endogenous Estrogens, Estrogen Metabolites, and Breast Cancer Risk in Postmenopausal Chinese Women." *JNCI: Journal of the National Cancer Institute* 108 (10). doi: 10.1093/jnci/djw103.

Morimoto, Y., S. M. Conroy, I. S. Pagano, M. Isaki, A. A. Franke, F. J. Nordt, and G. Maskarinec. 2012. "Urinary estrogen metabolites during a randomized soy trial." *Nutr Cancer* 64 (2):307-14. doi: 10.1080/01635581.2012.648819.

Mueck, AO, and H Seeger. 2010. "2-Methoxyestradiol—biology and mechanism of action." *Steroids* 75 (10):625-631.

Musey, V. C., D. C. Collins, D. R. Brogan, V. R. Santos, P. I. Musey, D. Martino-Saltzman, and J. R. Preedy. 1987. "Long term effects of a first pregnancy on the hormonal environment: estrogens and androgens." *J Clin Endocrinol Metab* 64 (1):111-8. doi: 10.1210/jcem-64-1-111.

Nakazawa, K., Y. Izumi, J. Fukuda, and T. Yasuda. 2006. "Hepatocyte spheroid culture on a polydimethylsiloxane chip having microcavities." *Journal of Biomaterials Science-Polymer Edition* 17 (8):859-873. doi: 10.1163/156856206777996853.

Nicholson, K. M., M. C. Bibby, and R. M. Phillips. 1997. "Influence of drug exposure parameters on the activity of paclitaxel in multicellular spheroids." *European Journal of Cancer* 33 (8):1291-1298. doi: https://doi.org/10.1016/S0959-8049(97)00114-7.

Nugoli, Mélanie, Paul Chuchana, Julie Vendrell, Béatrice Orsetti, Lisa Ursule, Catherine Nguyen, Daniel Birnbaum, Emmanuel JP Douzery, Pascale Cohen, and Charles Theillet. 2003. "Genetic variability in MCF-7 sublines: evidence of rapid genomic and RNA expression profile modifications." *BMC cancer* 3 (1):13.

Ortmann, O., and C. Lattrich. 2012. "The treatment of climacteric symptoms." *Desch. Arztebl. Int.* 109 (17):316-323. doi: 10.3238/arztebl.2012.0316.

Osborne, C Kent, Kimberly Hobbs, and Jeffrey M Trent. 1987. "Biological differences among MCF-7 human breast cancer cell lines from different laboratories." *Breast Cancer Res. Treat.* 9 (2):111-121.

Overk, C. R., P. Yao, L. R. Chadwick, D. Nikolic, Y. Sun, M. A. Cuendet, Y. Deng, A. S. Hedayat, G. F. Pauli, N. R. Farnsworth, R. B. van Breemen, and J. L. Bolton. 2005. "Comparison of the in vitro estrogenic activities of compounds from hops (*Humulus lupulus*) and red clover (*Trifolium pratense*)." *J. Agric. Food Chem.* 53 (16):6246-53. doi: 10.1021/jf050448p.

Overk, Cassia R, Ping Yao, Lucas R Chadwick, Dejan Nikolic, Yongkai Sun, Muriel A Cuendet, Yunfan Deng, AS Hedayat, Guido F Pauli, and Norman R Farnsworth. 2005. "Comparison of the in vitro estrogenic activities of compounds from hops (Humulus lupulus) and red clover (Trifolium pratense)." *Journal of agricultural and food chemistry* 53 (16):6246-6253.

Park, S. A., M. H. Lee, H. K. Na, and Y. J. Surh. 2016. "4-Hydroxyestradiol induces mammary epithelial cell transformation through Nrf2-mediated heme oxygenase-1 overexpression." *Oncotarget* 8 (1):164-178.

Park, Sin-Aye, Hye-Kyung Na, and Young-Joon Surh. 2012. "Resveratrol suppresses 4-hydroxyestradiol-induced transformation of human breast epithelial cells by blocking IκB kinaseβ-NF-κB signalling." *Free Radic. Res.* 46 (8):1051-1057.

Peterson, T Greg, Guo-Ping Ji, Marion Kirk, Lori Coward, Charles N Falany, and Stephen Barnes. 1998. "Metabolism of the isoflavones genistein and biochanin A in human breast cancer cell lines." *Am. J. Clin. Nutr.* 68 (6):1505-1511.

Peterson, T. G., L. Coward, M. Kirk, C. N. Falany, and S. Barnes. 1996. "The role of metabolism in mammary epithelial cell growth inhibition by the isoflavones genistein and biochanin A." *Carcinogenesis* 17 (9):1861-9.

Petrulis, J. R., G. Chen, S. Benn, J. LaMarre, and N. J. Bunce. 2001. "Application of the ethoxyresorufin-O-deethylase (EROD) assay to mixtures of halogenated aromatic compounds." *Environ Toxicol* 16 (2):177-84.

Piersen, C. E. 2003. "Phytoestrogens in botanical dietary supplements: implications for cancer." *Integr Cancer Ther* 2 (2):120-38. doi: 10.1177/1534735403002002004.

Piersen, CE, NL Booth, Y Sun, W Liang, JE Burdette, RB v Breemen, SE Geller, C Gu, S Banuvar, and LP Shulman. 2004. "Chemical and biological characterization and clinical evaluation of botanical dietary supplements: a phase I red clover extract as a model." *Curr. Med. Chem.* 11 (11):1361-1374.

Pisha, Emily, and John M Pezzuto. 1997. "Cell-based assay for the determination of estrogenic and anti-estrogenic activities." *Methods in cell Science* 19 (1):37-43.

Pitkin, J. 2012. "Alternative and complementary therapies for the menopause." *Menopause international* 18 (1):20-7. doi: 10.1258/mi.2012.012001.

Poluzzi, E., C. Piccinni, E. Raschi, A. Rampa, M. Recanatini, and F. De Ponti. 2014. "Phytoestrogens in postmenopause: the state of the art from a chemical, pharmacological and regulatory perspective." *Current Med. Chem.* 21 (4):417-36.

Powles, Trevor J, Anthony Howell, D Gareth Evans, Eugene V McCloskey, Sue Ashley, Rosemary Greenhalgh, Jenny Affen, Lesley Ann Flook, and Alwynne Tidy. 2008. "Red clover isoflavones are safe and well tolerated in women with a family history of breast cancer." *Menopause Int.* 14 (1):6-12.

Pritchett, L. E., K. M. Atherton, E. Mutch, and D. Ford. 2008. "Glucuronidation of the soyabean isoflavones genistein and daidzein by human liver is related to levels of UGT1A1 and UGT1A9 activity and alters isoflavone response in the MCF-7 human breast cancer cell line." *J Nutr Biochem* 19 (11):739-45. doi: 10.1016/j.jnutbio.2007.10.002.

Pruthi, Sandhya, Li Yang, Nicole P. Sandhu, James N. Ingle, Cheryl L. Beseler, Vera J. Suman, Ercole L. Cavalieri, and Eleanor G. Rogan. 2012. "Evaluation of serum estrogen-DNA adducts as potential biomarkers for breast cancer risk." *The Journal of Steroid Biochemistry and Molecular Biology* 132 (1â€“2):73-79. doi: http://dx.doi.org/10.1016/j.jsbmb.2012.02.002.

Raghavan, S., P. Mehta, E. N. Horst, M. R. Ward, K. R. Rowley, and G. Mehta. 2016. "Comparative analysis of tumor spheroid generation techniques for differential in vitro drug toxicity." *Oncotarget* 7 (13):16948-61. doi: 10.18632/oncotarget.7659.

Ramos Alvarenga, R. F., J. B. Friesen, D. Nikolic, C. Simmler, J. G. Napolitano, R. van Breemen, D. C. Lankin, J. B. McAlpine, G. F. Pauli, and S. N. Chen. 2014. "K-Targeted Metabolomic Analysis Extends Chemical Subtraction to DESIGNER Extracts: Selective Depletion of Extracts of Hops (*Humulus lupulus*)." *Journal of Natural Products* 77 (12):2595-2604. doi: 10.1021/np500376g.

Reding, Kerryn W, Muhammad Zahid, Ercole Cavalieri, Eleanor G Rogan, Brianne S Raccor, Charlotte Atkinson, Mellissa Yong, Katherine M Newton, and Johanna W Lampe. 2014. "Associations between dietary intake of fruits and vegetables in relation to urinary estrogen DNA adduct ratio." *Open J. Prev. Med.* 4 (6):429.

Reynolds, D. S., K. M. Tevis, W. A. Blessing, Y. L. Colson, M. H. Zaman, and M. W. Grinstaff. 2017. "Breast Cancer Spheroids Reveal a Differential Cancer Stem Cell Response to Chemotherapeutic Treatment." *Sci Rep* 7 (1):10382. doi: 10.1038/s41598-017-10863-4.

Roberts, D. W., D. R. Doerge, M. I. Churchwell, G. Gamboa da Costa, M. M. Marques, and W. H. Tolleson. 2004. "Inhibition of extrahepatic human cytochromes P450 1A1 and 1B1 by metabolism of isoflavones found in Trifolium pratense (red clover)." *J Agric Food Chem* 52 (21):6623-32. doi: 10.1021/jf049418x.

Rossouw, J. E., G. L. Anderson, R. L. Prentice, A. Z. LaCroix, C. Kooperberg, M. L. Stefanick, R. D. Jackson, S. A. A. Beresford, B. V. Howard, K. C. Johnson, J. M. Kotchen, and J. Ockene. 2002a. "Risks and benefits of estrogen plus progestin in healthy postmenopausal women - Principal results from the Women's Health Initiative randomized controlled trial." *Jama-Journal of the American Medical Association* 288 (3):321-333.

Rossouw, J. E., G. L. Anderson, R. L. Prentice, A. Z. LaCroix, C. Kooperberg, M. L. Stefanick, R. D. Jackson, S. A. Beresford, B. V. Howard, K. C. Johnson, J. M. Kotchen, and J. Ockene. 2002b. "Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial." *JAMA* 288:321 - 333.

Rossouw, J. E., R. L. Prentice, J. E. Manson, L. Wu, D. Barad, V. M. Barnabei, M. Ko, A. Z. LaCroix, K. L. Margolis, and M. L. Stefanick. 2007. "Postmenopausal hormone therapy and risk of cardiovascular disease by age and years since menopause." *JAMA* 297 (13):1465-477.

Roush, K. 2012. "Managing menopausal symptoms." *Am. J. Nurs.* 112 (6):28-35. doi: 10.1097/01.NAJ.0000415120.76982.7a.

Safe, S., M. Wormke, and I. Samudio. 2000. "Mechanisms of inhibitory aryl hydrocarbon receptor-estrogen receptor crosstalk in human breast cancer cells." *J. Mammary Gland Biol. Neoplasia* 5 (3):295-306.

Sakai, Y., T. Tanaka, J. Fukuda, and K. Nakazawa. 2010. "Alkoxyresorufin O-dealkylase assay using a rat hepatocyte spheroid microarray." *Journal of Bioscience and Bioengineering* 109 (4):395-399. doi: 10.1016/j.jbiosc.2009.10.001.

Sampson, Joshua N, Roni T Falk, Catherine Schairer, Steven C Moore, Barbara J Fuhrman, Cher M Dallal, Douglas C Bauer, Joanne F Dorgan, Xiao-Ou Shu, and Wei Zheng. 2017. "Association of estrogen metabolism with breast cancer risk in different cohorts of postmenopausal women." *Cancer Res.* 77 (4):918-925.

Sampson, Joshua N., Roni T. Falk, Catherine Schairer, Steven C. Moore, Barbara J. Fuhrman, Cher M. Dallal, Douglas C. Bauer, Joanne F. Dorgan, Xiao-Ou Shu, Wei Zheng, Louise A. Brinton, Mitchell H. Gail, Regina G. Ziegler, Xia Xu, Robert N. Hoover, and Gretchen L. Gierach. 2017. "Association of Estrogen Metabolism with Breast Cancer Risk in Different Cohorts of Postmenopausal Women." *Cancer Research* 77 (4):918-925. doi: 10.1158/0008-5472.Can-16-1717.

Sasano, H., Y. Miki, S. Nagasaki, and T. Suzuki. 2009. "In situ estrogen production and its regulation in human breast carcinoma: from endocrinology to intracrinology." *Pathol Int* 59 (11):777-89. doi: 10.1111/j.1440-1827.2009.02444.x.

Sasano, H., S. Nagasaki, Y. Miki, and T. Suzuki. 2009. "New developments in intracrinology of human breast cancer: estrogen sulfatase and sulfotransferase." *Ann N Y Acad Sci* 1155:76-9. doi: 10.1111/j.1749-6632.2008.03683.x.

Scott, L. M., P. Durant, S. Leone-Kabler, C. E. Wood, T. C. Register, A. Townsend, and J. M. Cline. 2008. "Effects of prior oral contraceptive use and soy isoflavonoids on estrogen-metabolizing cytochrome P450 enzymes." *The Journal of steroid biochemistry and molecular biology* 112 (4-5):179-185. doi: 10.1016/j.jsbmb.2008.10.001.

Setchell, KD. 1998. "Phytoestrogens: the biochemistry, physiology, and implications for human health of soy isoflavones." *The American journal of clinical nutrition* 68 (6):1333S-1346S.

Setchell, Kenneth DR, Nadine M Brown, Pankaj Desai, Linda Zimmer-Nechemias, Brian E Wolfe, Wayne T Brashear, Abby S Kirschner, Aedin Cassidy, and James E Heubi. 2001. "Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements." *The Journal of nutrition* 131 (4):1362S-1375S.

Shiau, Andrew K., Danielle Barstad, Paula M. Loria, Lin Cheng, Peter J. Kushner, David A. Agard, and Geoffrey L. Greene. 1998. "The Structural Basis of Estrogen Receptor/Coactivator Recognition and the Antagonism of This Interaction by Tamoxifen." *Cell* 95 (7):927-937. doi: 10.1016/S0092-8674(00)81717-1.

Shimada, T., H. Yamazaki, M. Foroozesh, N. E. Hopkins, W. L. Alworth, and F. P. Guengerich. 1998. "Selectivity of polycyclic inhibitors for human cytochrome P450s 1A1, 1A2, and 1B1." *Chem Res Toxicol* 11 (9):1048-56. doi: 10.1021/tx980090+ [doi]tx980090+ [pii].

Shull, J. D., T. J. Spady, M. C. Snyder, S. L. Johansson, and K. L. Pennington. 1997. "Ovary-intact, but not ovariectomized female ACI rats treated with 17 beta estradiol rapidly develop mammary carcinoma." *Carcinogenesis* 18 (8):1595-1601. doi: 10.1093/carcin/18.8.1595.

Siegel, R. L., K. D. Miller, and A. Jemal. 2016a. "Cancer Statistics, 2016." *Ca-a Cancer Journal for Clinicians* 66 (1):7-30. doi: 10.3322/caac.21332.

Siegel, Rebecca L, Kimberly D Miller, and Ahmedin Jemal. 2016b. "Cancer statistics, 2016." *CA Cancer J. Clin.* 66 (1):7-30.

Siegel, Rebecca L, Kimberly D Miller, and Ahmedin Jemal. 2016c. "Cancer statistics, 2016." *CA: a cancer journal for clinicians* 66 (1):7-30.

Smith, T., Kawa, K., Eckl, V., Morton, C., Stredney, R. 2018. "Herbal Supplement Sales in US Increased 8.5% in 2017, Topping $8 Billion." *HerbalGram* (119):62-71.

Snelten, Courtney S, Birgit Dietz, and Judy L Bolton. 2012. "Modulation of estrogen chemical carcinogenesis by botanical supplements used for postmenopausal women's health." *Drug Discov. Today Dis. Mech.* 9 (1):47-54.

Sotoca, A. M., H. van den Berg, J. Vervoort, P. van der Saag, A. Strom, J. A. Gustafsson, I. Rietjens, and A. J. Murk. 2008. "Influence of cellular ERalpha/ERbeta ratio on the ERalpha-agonist induced proliferation of human T47D breast cancer cells." *Toxicol Sci* 105 (2):303-11. doi: 10.1093/toxsci/kfn141.

Spink, D. C. , B.C. Spink, J. Q. Cao, J.A. DePasquale, B.T. Pentecost, M. J. Fasco, Y. Li, and Sutter T. R. 1998. "Differential expression of CYP1A1 and CYP1B1 in

human breast epithelial cells and breast tumor cells." *Carcinogenesis* 19 (2):291–298.

Spink, D. C., B. C. Spink, J. Q. Cao, J. A. DePasquale, B. T. Pentecost, M. J. Fasco, Y. Li, and T. R. Sutter. 1998. "Differential expression of CYP1A1 and CYP1B1 in human breast epithelial cells and breast tumor cells." *Carcinogenesis* 19 (2):291-8. doi: 10.1093/carcin/19.2.291.

Spink, David C, Barbara H Katz, Mirza M Hussain, Brian T Pentecost, Zhimin Cao, and Barbara C Spink. 2003. "Estrogen regulates Ah responsiveness in MCF-7 breast cancer cells." *Carcinogenesis* 24 (12):1941-1950.

Sweeney, Elizabeth E., Ping Fan, and V. Craig Jordan. 2014. "Mechanisms underlying differential response to estrogen-induced apoptosis in long-term estrogen-deprived breast cancer cells." *International journal of oncology* 44 (5):1529-1538. doi: 10.3892/ijo.2014.2329.

Taechakraichana, N., U. Jaisamrarn, K. Panyakhamlerd, S. Chaikittisilpa, and K. K. Limpaphayom. 2002. "Climacteric: concept, consequence and care." *J. Med. Assoc. Thai.* 85 Suppl 1:S1-15.

Touillaud, M., A. Gelot, S. Mesrine, C. Bennetau-Pelissero, F. Clavel-Chapelon, P. Arveux, F. Bonnet, M. Gunter, M. C. Boutron-Ruault, and A. Fournier. 2019. "Use of dietary supplements containing soy isoflavones and breast cancer risk among women aged >50 y: a prospective study." *Am J Clin Nutr* 109 (3):597-605. doi: 10.1093/ajcn/nqy313.

Tsuchiya, Yuki, Miki Nakajima, Satoru Kyo, Taro Kanaya, Masaki Inoue, and Tsuyoshi Yokoi. 2004a. "Human CYP1B1 is regulated by estradiol via estrogen receptor." *Cancer Res.* 64 (9):3119-3125.

Tsuchiya, Yuki, Miki Nakajima, Satoru Kyo, Taro Kanaya, Masaki Inoue, and Tsuyoshi Yokoi. 2004b. "Human CYP1B1 Is Regulated by Estradiol via Estrogen Receptor." *Cancer Res* 64 (9):3119-3125. doi: 10.1158/0008-5472.can-04-0166.

Uifălean, Alina, Anca Farcaş, Maria Ilieş, Simona Codruţa Hegheş, Corina Ionescu, and Cristina Adela Iuga. 2015. "Assessment of isoflavone aglycones variability in soy food supplements using a validated HPLC-UV method." *Clujul medical (1957)* 88 (3):373-380. doi: 10.15386/cjmed-468.

van Breemen, R. B., Y. Yuan, S. Banuvar, L. P. Shulman, X. Qiu, R. F. R. Alvarenga, S. N. Chen, B. M. Dietz, J. L. Bolton, G. F. Pauli, E. Krause, M. Viana, and D. Nikolic. 2014. "Pharmacokinetics of prenylated hop phenols in women following oral administration of a standardized extract of hops." *Molecular Nutrition & Food Research* 58 (10):1962-1969. doi: 10.1002/mnfr.201400245.

Van Breemen, Richard B. 2015. "Development of safe and effective botanical dietary supplements." *Journal of Medicinal Chemistry* 58 (21):8360-8372.

van Landeghem, A. A., J. Poortman, M. Nabuurs, and J. H. Thijssen. 1985. "Endogenous concentration and subcellular distribution of estrogens in normal and malignant human breast tissue." *Cancer Res* 45 (6):2900-6.

Wagner, Jorg, Ling Jiang, and Leane Lehmann. 2008. "Phytoestrogens modulate the expression of 17a-estradiol metabolizing enzymes in cultured MCF-7 cells." *Advances in experimental medicine and biology* 617:625-632.

Wall, R. J., G. He, M. S. Denison, C. Congiu, V. Onnis, A. Fernandes, D. R. Bell, M. Rose, J. C. Rowlands, G. Balboni, and I. R. Mellor. 2012. "Novel 2-amino-isoflavones exhibit aryl hydrocarbon receptor agonist or antagonist activity in a species/cell-specific context." *Toxicology* 297 (1-3):26-33. doi: 10.1016/j.tox.2012.03.011.

Wang, S., T. L. Dunlap, C. E. Howell, O. C. Mbachu, E. A. Rue, R. Phansalkar, S. N. Chen, G. F. Pauli, B. M. Dietz, and J. L. Bolton. 2016a. "Hop (Humulus lupulus L.) Extract and 6-Prenylnaringenin Induce P450 1A1 Catalyzed Estrogen 2-Hydroxylation." *Chem Res Toxicol* 29 (7):1142-50. doi: 10.1021/acs.chemrestox.6b00112.

Wang, Shuai, Tareisha L Dunlap, Caitlin E Howell, Obinna C Mbachu, Emily A Rue, Rasika Phansalkar, Shao-Nong Chen, Guido F Pauli, Birgit M Dietz, and Judy L

Bolton. 2016b. "Hop (Humulus lupulus L.) extract and 6-prenylnaringenin induce P450 1A1 catalyzed estrogen 2-hydroxylation." *Chemical research in toxicology* 29 (7):1142-1150.

Wang, Shuai, Tareisha L. Dunlap, Lingyi Huang, Yang Liu, Charlotte Simmler, Daniel D. Lantvit, Jenna Crosby, Caitlin E. Howell, Huali Dong, Shao-Nong Chen, Guido F. Pauli, Richard B. van Breemen, Birgit M. Dietz, and Judy L. Bolton. 2018. "Evidence for Chemopreventive and Resilience Activity of Licorice: Glycyrrhiza Glabra and G. Inflata Extracts Modulate Estrogen Metabolism in ACI Rats." *Cancer Prevention Research* 11 (12):819-830. doi: 10.1158/1940-6207.Capr-18-0178.

Wang, X. Y., G. N. Nie, H. Y. Yang, and L. L. Zong. 2011. "Chinese medicine for menopausal syndrome: current status, problems and strategies." *Chin. J. Integr. Med.* 17 (12):889-92. doi: 10.1007/s11655-011-0931-8.

Weller, Michael G. 2012. "A unifying review of bioassay-guided fractionation, effect-directed analysis and related techniques." *Sensors (Basel, Switzerland)* 12 (7):9181-9209. doi: 10.3390/s120709181.

Wen, W., Z. Ren, X. O. Shu, Q. Cai, C. Ye, Y. T. Gao, and W. Zheng. 2007. "Expression of cytochrome P450 1B1 and catechol-O-methyltransferase in breast tissue and their associations with breast cancer risk." *Cancer Epidemiol Biomarkers Prev* 16 (5):917-20. doi: 10.1158/1055-9965.Epi-06-1032.

Wu, F. J., J. R. Friend, R. P. Remmel, F. B. Cerra, and W. S. Hu. 1999. "Enhanced cytochrome P450IA1 activity of self-assembled rat hepatocyte spheroids." *Cell Transplantation* 8 (3):233-246. doi: 10.1177/096368979900800304.

Xu, X., A. M. Duncan, B. E. Merz, and M. S. Kurzer. 1998. "Effects of soy isoflavones on estrogen and phytoestrogen metabolism in premenopausal women." *Cancer Epidemiol Biomarkers Prev* 7 (12):1101-8.

Xu, X., A. M. Duncan, K. E. Wangen, and M. S. Kurzer. 2000. "Soy consumption alters endogenous estrogen metabolism in postmenopausal women." *Cancer Epidemiol Biomarkers Prev* 9 (8):781-6.

Yager, J. D., and N. E. Davidson. 2006a. "Estrogen carcinogenesis in breast cancer." *N. Engl. J. Med.* 354 (3):270-82. doi: 10.1056/NEJMra050776.

Yager, James D. 2015. "Mechanisms of estrogen carcinogenesis: The role of E2/E1–quinone metabolites suggests new approaches to preventive intervention–A review." *Steroids* 99:56-60.

Yager, James D., and Nancy E. Davidson. 2006b. "Estrogen Carcinogenesis in Breast Cancer." *N Engl J Med* 354 (3):270-282. doi: doi:10.1056/NEJMra050776.

Yaghjyan, Lusine, and Graham A. Colditz. 2011. "Estrogens in the breast tissue: a systematic review." *Cancer causes & control : CCC* 22 (4):529-540. doi: 10.1007/s10552-011-9729-4.

Yang, Chun Z., Warren Casey, Matthew A. Stoner, Gayathri J. Kollessery, Amy W. Wong, and George D. Bittner. 2014. "A robotic MCF-7:WS8 cell proliferation assay to detect agonist and antagonist estrogenic activity." *Toxicological sciences : an official journal of the Society of Toxicology* 137 (2):335-349. doi: 10.1093/toxsci/kft250.

Yang, L., M. Zahid, Y. Liao, E. G. Rogan, E. L. Cavalieri, N. E. Davidson, J. D. Yager, K. Visvanathan, J. D. Groopman, and T. W. Kensler. 2013. "Reduced formation of depurinating estrogen-DNA adducts by sulforaphane or KEAP1 disruption in human mammary epithelial MCF-10A cells." *Carcinogenesis* 34 (11):2587-92. doi: 10.1093/carcin/bgt246.

Zhang, Shu, Chunhua Qin, and Stephen H Safe. 2003. "Flavonoids as aryl hydrocarbon receptor agonists/antagonists: effects of structure and cell context." *Environ. Health Perspect.* 111 (16):1877.

Zhao, Zibo, Lu Wang, Taryn James, Youngeun Jung, Ikyon Kim, Renxiang Tan, F. Michael Hoffmann, and Wei Xu. 2015. "Reciprocal Regulation of ERα and ERβ Stability and Activity by Diptoindonesin G." *Chemistry & biology* 22 (12):1608-1621. doi: 10.1016/j.chembiol.2015.10.011.

Ziegler, R. G., B. J. Fuhrman, S. C. Moore, and C. E. Matthews. 2015. "Epidemiologic studies of estrogen metabolism and breast cancer." *Steroids* 99 (Pt A):67-75. doi: 10.1016/j.steroids.2015.02.015.

APPENDIX

UIC

Caitlin Howell <chowel5@uic.edu>

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VITA

Caitlin E. Howell

Education

2014-present PhD, Pharmacognosy, University of Illinois at Chicago, Chicago, IL.
Advisor: Judy Bolton

2008-2012 BS, Chemistry, University of South Florida, Tampa, FL

Research Experience

University of Illinois at Chicago College of Pharmacy in Chicago, IL (2015-present)

Advisor: Judy Bolton, PhD

Description: Within UIC/NIH Center for Botanical Dietary Supplements Research, investigated topics related to botanical modulation of estrogen carcinogenesis

- Utilized LC-MS/MS, fluorescence imaging, luminescence imaging, colorimetric imaging, qPCR, fluorescence quantification to investigate the effects of different botanicals on the estrogen oxidative metabolism.
- Developed a new screening assay to measure activity of P450 1A1/1B1.
- Developed computational binding models of enzymes involved in estrogen oxidative metabolism.

- Investigated effect of estrogen on the modulation of estrogen oxidative metabolism by isoflavones
- Mentored six (1 undergrad, 2 PharmD, 1 master's, 2 Ph.D.) students in topics such as cell culture, experimental design, assay methods, and scientific reasoning.

University of South Florida College of Pharmacy in Tampa, FL (2011- 2012)

Advisors: Jiazhi Sun, MD and Shufeng Zhou, MD, PhD

Description: Assisted in drug discovery and repositioning research by docking, analyzing binding sites, running assays, culturing cells.

University of South Florida Honors College (2011-2012)

Advisor: Kenneth Caswell, PhD

Description: Researched chemistry education, specifically how to incorporate chemistry into the education of elementary and middle students by connecting mathematics to chemistry concepts.

Paper: Thesis Entitled "Quelling Fear of Chemistry: A Method Designed for the Incorporation of General Chemistry concepts into the Education of Elementary and Middle School Students"

Thesis honored as an Outstanding Thesis for 2012 and can be accessed at http://scholarcommons.usf.edu/honors_et/81/

University of South Florida Robotics Engineering Department (August 2008-December 2008)

Advisor: Cindy Bethel, PhD

Description: Assisted in researching how different components of search and rescue robots affected the conscious victims of disaster situations.

Teaching Experiences

2015-present	Research assistant, University of Illinois at Chicago
2014 and 2016	Teaching assistant, University of Illinois at Chicago
2014-2014	High school/middle school tutor, Learning Resource Center, Lakeland, FL
2012 -2013	English/homeroom teacher in various positions in eastern China
2011-2012	Peer Leader (Calculus), University of South Florida
2010 -2012	STEM Tutor, University of South Florida
2010-2010	Volunteer English Teacher, Various locations Huili County, Sichuan, China, Arranged through Tsinghua University, Beijing, China
2005 -2011	Volunteer Math Tutor, East Area Adult School, Auburndale, Florida

Publications

1. Wang S., Dunlap T., Huang L., Liu Y., Simmler C., Lantvit D., Crosby J., **Howell C.,** Dong H., Chen S., Pauli G., van-Breemen R., Dietz B., Bolton J. Evidence for Chemopreventive and Resilience Activity of Licorice: *Glycyrrhiza Glabra* and G. *Inflata* Extracts Modulate Estrogen Metabolism in ACI Rats. *Cancer Prev. Res.* **2018** 11(12):819-830.
2. **Howell C**., Dunlap T., Mukand N., Chen S., Pauli G., Dietz B., Bolton J. Red Clover Aryl Hydrocarbon Receptor (AhR) and Estrogen Receptor (ER) Agonists

Enhance Genotoxic Estrogen Metabolism. *Chem. Res. Toxicol.* **2017** *30* (11), 2084-2092. (Co-first Author)

3. Wang S., Dunlap T., **Howell C**., Mbachu O., Rue E., Phansalkar R., Chen S., Pauli G., Dietz B., Bolton J. Hop (Humulus lupulus L.) extract and 6-Prenylnaringenin induce P450 1A1 catalyzed estrogen 2-hydroxylation*. Chem. Res. Toxicol*. **2016** 29 (7), 1142–1150.
4. Wang Z., Sun J., **Howell C.**, Zhou Q., He Z., Yang T., Chew H., Duan W., Zhou Z., Kanwar J., Zhou S. Prediction of the likelihood of drug interactions with kinase inhibitors based on *in vitro* and computational studies. *Fundam Clin Pharmacol*. **2014** 28 (5), 551-82.

Poster presentations

1. **CARBON Meeting:** Howell,C., Huali D., Hitzman, R., Youn, I., S. Chen, S., Pauli, G., Dietz, B., Bolton J. Influence of soy and its phytoestrogens on estrogen metabolism in breast cancer cells is dependent on the presence of estrogen CARBON Meeting, Bethesda, MD, May 2019
2. **MIKI Meeting:** Howell,C., Hitzman, R., Dunlap, T., Dietz, B., Bolton J. Spheroids as a model for P450 1A1/1B1 activity. MIKI Meeting, Chicago, IL, April 2018
3. **UIC College of Pharmacy Research Day:** Howell,C., Hitzman, R., Dunlap, T., Dietz, B., Bolton J. Spheroids as a model for P450 1A1/1B1 activity. UIC College of Pharmacy Research Day. Chicago, IL February 2018

4. **CARBON Meeting:** Howell, C., Dunlap, T., Dietz, B., Bolton J., Are spheroids a better model for predicting P450 1A1/1B1 activity?. CARBON Meeting. Chicago, IL, May 2017
5. **MIKI Meeting:** Howell, C., Dunlap, T., Wang, S. Chen, S., Pauli, G., Dietz, B., Bolton J.,Modulation of the estrogen chemical carcinogenesis pathway by red clover and bioactive phytoestrogens: effect of E_2 on AhR gene induction. MIKI Meeting, Minneapolis, MN, April 2017
6. **UIC College of Pharmacy Research Day:** Howell, C., Dunlap, T., Wang, S. Chen, S., Pauli, G., Dietz, B., Bolton J. Modulation of the estrogen chemical carcinogenesis pathway by red clover and bioactive phytoestrogens: effect of E_2 on AhR gene induction.
7. **Great Lakes Drug Metabolism and Disposition Group Meeting:** Howell, C., Dunlap, T., Wang, S. Chen, S., Pauli, G., Dietz, B., Bolton J. Red clover and the arylhydrocarbon receptor (AhR) ligands biochanin A and formononetin modulate P450 1B1 and estrogen metabolism. Great Lakes Drug Metabolism and Disposition Group Meeting. Chicago, IL May 2016.
8. **CARBON Meeting:** Howell, C., Dunlap, T., Wang, S. Chen, S., Pauli, G., Dietz, B., Bolton J., Red clover and the arylhydrocarbon receptor (AhR) ligands biochanin A and formononetin modulate P450 1B1 and estrogen metabolism. CARBON Meeting. Bethesda, MD, April 2016
9. **MIKI Meeting:** Howell, C., Dunlap, T., Wang, S. Chen, S., Pauli, G., Dietz, B., Bolton J., Red clover and the arylhydrocarbon receptor (AhR) ligands biochanin

A and formononetin modulate P450 1B1 and estrogen metabolism. MIKI Meeting, Iowa City, IA, April 2016

10. **UIC College of Pharmacy Research Day:** Howell, C., Dunlap, T., Wang, S. Chen, S., Pauli, G., Dietz, B., Bolton J., Red clover and the arylhydrocarbon receptor (AhR) ligands biochanin A and formononetin modulate P450 1B1 and estrogen metabolism. UIC College of Pharmacy Research Day, Chicago, IL, USA, Chicago, IL, February 2016
11. **Raymond N. Castle Student Research Conference:** Howell, C., Jiazhi, S., Zhou, S., Sneed, K. Computational identification and characterization of off-target TKI binding. Raymond N. Castle Student Research Conference, Tampa, FL, April 2012. Awards: 2nd Place in Undergraduate Biochemistry Category.

Awards and honors

2018 Van Doren Scholar award, UIC College of Pharmacy, Chicago, IL.

2012 2nd Place at Castle Conference, University of South Florida

2012 University Scholar

2012 Outstanding Thesis Spring 2012

Affiliations/memberships

2018-present Member of the American Chemical Society

2018-2019 Treasurer of Ju Jitsu Club

2014-present Member of MCP Graduate Student Council

2014-present Member of the UIC Graduate Student Council