Prevention of Estrogen Carcinogenesis by Botanicals, SERMs and NO/cGMP Pathway

Modulators

By

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THESIS

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This thesis is dedicated to my husband Kasun and daughter Methuli.
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<th>Abbreviation</th>
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<tbody>
<tr>
<td>AI</td>
<td>Aromatase Inhibitor</td>
</tr>
<tr>
<td>BT-SERM</td>
<td>Benzothiophene SERMs</td>
</tr>
<tr>
<td>BDS</td>
<td>Botanical dietary supplements</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic GMP</td>
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<tr>
<td>COMT</td>
<td>Catechol-o-methyl transferase</td>
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<tr>
<td>DMA</td>
<td>Desmethy zaroxifene</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>E₂</td>
<td>17β-Estradiol</td>
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<tr>
<td>ER</td>
<td>Estrogen receptor</td>
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<tr>
<td>ERE</td>
<td>Estrogen Response Elements</td>
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<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
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<tr>
<td>F-DMA</td>
<td>4'-Fluoro DMA</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>HRT</td>
<td>Hormone replacement therapy</td>
</tr>
<tr>
<td>ICI</td>
<td>ICI182780, Fulvestrant</td>
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<tr>
<td>KT</td>
<td>KT5823</td>
</tr>
<tr>
<td>L-NAME</td>
<td>L-Nitro-Z-arginine methylester</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MTT</td>
<td>3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase, (i-inducible, e-endothelial, n-neuronal)</td>
</tr>
<tr>
<td>NQO1</td>
<td>NAD(P)H quinone oxidoreductase 1</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>ODQ</td>
<td>1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>8-PN</td>
<td>8-Prenylnaringenin</td>
</tr>
<tr>
<td>PKG</td>
<td>cGMP-dependent protein kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>SERMs</td>
<td>Selective estrogen receptor modulators</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering ribonucleic acid</td>
</tr>
<tr>
<td>sGC</td>
<td>Soluble guanylyl cyclase</td>
</tr>
<tr>
<td>XH</td>
<td>Xanthohumol</td>
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SUMMARY

Breast cancer is a major hormone dependent cancer which is the second leading cause of cancer death among women. In order to discover new preventive measures for breast cancer, it is important to understand the mechanisms of estrogen carcinogenesis. Estrogen induced cell proliferation in estrogen receptor positive cells (hormonal pathway) and formation of reactive estrogen quinoids mediated by cytochrome P450s (chemical pathway) are believed to contribute to estrogen carcinogenesis. Estrogens are oxidized to the catechols, 2-hydroxyestradiol (2-OHE$_2$) and 4-hydroxyestradiol (4-OHE$_2$) by P450 1A1/1A2 and P450 1B1, respectively. Both catechols are further oxidized to form electrophilic o-quinones which can react with DNA and proteins; however, 4-hydroxyestradiol-o-quinone (4-OHE$_2$-Q) is considered the ultimate carcinogen. In order to examine estrogen chemical carcinogenesis, ER negative, immortalized human breast epithelial (MCF-10A) cells were used as a model system in the present study. Oxidative estrogen metabolism was studied by measuring/quantifying estrogen metabolites using LC/MS-MS and monitoring the formation of reactive oxygen species using confocal microscopy. Cellular transformation of normal breast epithelial cells to a malignant phenotype was measured using anchorage independent colony formation assay. Attenuation of estrone methyl ether formation, E$_2$ induced ROS formation and malignant transformation could serve as potential biomarkers in estrogen chemical carcinogenesis in vitro.

Plant extracts have been extensively used as dietary supplements for women’s health, particularly for menopausal symptom relief. Among the botanicals tested in the current study, hops (Humulus lupulus) significantly prevented estrogen induced cellular transformation of MCF-10A cells. Oxidative estrogen metabolism was attenuated by hops through down regulation of CYP450 1B1 and 1A1. Xanthohumol (XH) and 8-prenylnaringenin (8-PN) which are isolated compounds from hops, were tested for their effect on estrogen metabolism and 8-PN showed a significant inhibition on estrone methyl ether formation. Also 8-PN was a potent inhibitor of malignant transformation at nanomolar concentrations. Although XH did
not reduce oxidative estrogen metabolism, it did reduce estrogen induced malignant transformation suggesting that the estrogen carcinogenesis prevention by hops is mediated via different mechanisms.

Selective Estrogen Receptor Modulators (SERMs) have been used for chemoprevention and in the treatment of post menopausal osteoporosis. Among the SERMs tested, raloxifene (Ral) and desmethylarzoxifen (DMA) showed a significant reduction in catechol estrogen formation whereas 4'F-DMA had little effect suggesting that SERMs that can form a diquinone methide could attenuate estrogen metabolism. Estrogen induced oxidative stress and malignant transformation were also inhibited by Ral and DMA. SERMs mediated this inhibitory effect by inducing the formation of conjugative, phase II estrogen metabolites. In addition, the reactive metabolite of the prototypical SERM, tamoxifen did not inhibit estrogen metabolism and E₂ induced ROS formation in breast epithelial cells. These data suggest that Ral and DMA possess chemopreventive activity through inhibition of genotoxic estrogen quinone formation, in addition to their action via estrogen receptors.

Elevated nitric oxide synthase (NOS) is implicated in the patho-physiology of many tumors and has been linked to tumor status and outcome. In the present study inhibition of NOS with L-NAME showed a significant reduction in estrogen metabolism and estrogen induced malignant transformation in MCF-10A cells. Attenuation of NO/cGMP signaling pathway with ODQ (NO-GC inhibitor) and KT-5823 (PKG inhibitor) significantly inhibited the catechol estrogen formation. Blocking of malignant transformation by SERMs involves upregulation of detoxification enzymes, whereas the mechanism of NO/cGMP modulation does not.

In conclusion, this study demonstrates that estrogen chemical carcinogenesis is mediated via oxidative estrogen metabolism and can be analyzed by measuring estrone methyl ether production, ROS generation, and anchorage independent colony formation in human breast epithelial cells. Therefore these factors could serve as useful biomarkers in estrogen carcinogenesis and modulation/attenuation of estrogen carcinogenesis could be achieved via manipulating them. Among the compounds tested for
SUMMARY (continued)

chemopreventive properties, there was a significant inhibition in the oxidative estrogen metabolism and estrogen carcinogenesis with SERMs, hops, and NOS inhibitors. It was evident that although these compounds could attenuate estrogen chemical carcinogenesis, the mechanisms were different for each case. This is the first study to investigate the effect of SERMs, hops, and L-NAME on oxidative estrogen metabolism and its effect on human breast epithelial cells. Further investigation is needed to find out the effects of hops, DMA, Ral, and L-NAME towards estrogen carcinogenesis in an *in vivo* system.
1: INTRODUCTION

1.1. Breast cancer

Breast cancer is the most common cancer among women worldwide excluding skin cancers and is the second cause of cancer deaths among women exceeded by lung cancer (1). Breast cancer accounts for about 1 in 3 cancers diagnosed in US women (2, 3). The incidence of breast cancer depends on several factors such as age, race/ethnicity, family history of breast cancer, obesity, diet and life style (3, 4).

It has been reported that there is a high risk associated with increasing age of women until menopause (5). Previous epidemiological studies showed that there is an increased risk of breast cancer occurrence in the individuals who had late first child birth and a family history of breast cancer (6, 7). The connection between life style and breast cancer has also been studied and the diet, obesity and reduced physical exercise are known to associate with breast cancer risk (8, 9). Dramatic increased in breast cancer risk was observed with the Asian immigrants in USA compared to the women reside in their native countries. Exposure to different environmental factors and change in life style were the main factors responsible for increased breast cancer risk in Asian immigrants in USA (10, 11).

Growing body of evidence shows that there is a clear correlation between polymorphism and/ or mutations of breast cancer related genes and risk of breast cancer occurrence. BRCA (breast cancer suppressor gene) is a key tumor suppressor gene which exhibits hormone dependent pattern of expression in breast cancer. Women with loss of function mutations of one of the breast cancer susceptibility genes, BRCA1 or BRCA2 have an increased risk of developing breast cancer by the age of 70 (12). BRCA1 plays a major role in DNA repair and maintains the integrity of cellular genome. BRCA1 functions in the DNA repair mainly via homologous recombination and nucleotide excision. Since mutations in BRCA1 are associated with breast cancer risk, BRCA1 has become a therapeutic target for breast cancer (13). In addition to breast cancer, individuals with BRCA mutations are predisposed to increased risk of developing ovarian, prostate and pancreatic cancer (14). Another DNA repair enzyme, poly- (ADP ribose) polymerase (PARP) is also been studied rapidly due to its association with carcinogenesis. PARPs are
enzymes that are important in DNA repair, gene transcription, chromatin architecture and apoptosis in human cells (15). PARP1 is a widely studied enzyme due to its importance in single strand base excision repair. PARP inhibition leads to the accumulation of single strand DNA breaks and subsequent double strand breaks at the replication fork which will lead to induce apoptosis and cell death (16). A phase 2 clinical study done with BRCA1 and BRCA2 mutated advanced breast cancer patients showed that treatment with olparib, a potent oral PARP inhibitor could lead to 41% of objective tumor response in these patients (17). Since many effective cytotoxic chemotherapies and radiotherapies exert their anti tumor effect through DNA damage, inhibition of DNA repair with a PARP inhibitor could be used to sensitize tumor cells to DNA damaging chemotherapy (15).

More detailed classification of breast cancer may provide better directions for treatment and better understanding of disease state and prognosis. Depending on the area of origin of the cancer, breast cancer can be broadly classified into two groups; ductal and lobular carcinoma. Ductal carcinoma can be further divide into invasive ductal carcinoma and ductal carcinoma in situ; and lobular carcinoma is basically invasive according to the histopathological classification suggested by World Health Organization (WHO) (18). Breast cancer can also be classified into different stages considering the location of the primary tumor, tumor size and number of tumors, lymph node involvement and the presence and absence of metastasis (19). This is known as the TNM (Tumor, Node, Metastasis) staging and is an internationally used classification. Another widely used breast cancer classification is based on the receptor status of the tumor. There are three main receptors associated with breast cancer, i.e. estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor2 (HER2). Considering the receptor status, breast cancers can be broadly classified into few groups, such as; basal or triple negative [ER(-), PR(-), HER2 (-)], luminal A & B [ER (+)], luminal ER-/AR+ and ERBB2/HER2+ (20). Triple negative breast cancer is considered more aggressive compared to other types and associated with relatively poor prognosis. ER and other receptor expression in breast cancer is generally associated with better clinical outcome since most of the targeted breast cancer therapies can be applied successfully.
1.2. Estrogen carcinogenesis

In 1896, a British Physician, George Beatson demonstrated that oopharectomy (surgical removal of ovaries) of premenopausal women with breast cancer can reduce the tumor mass and dramatically improve the course of the disease (21). That was the first report to show that breast cancer is estrogen dependent and reduced exposure to estrogens can reduce the risk of developing the disease.

Increased exposure to endogenous estrogens can enhance the risk of developing breast cancer in women. Long term exposure to estrogens could be resulted from one or more reasons; such as early puberty, late menopause, late first child birth, family history of breast cancer and use of hormone replacement therapy (7, 11, 22-24). Mechanism of estrogen carcinogenesis can be explained by two pathways; i.e. ER mediated increased cell proliferation (hormonal pathway) and production of highly reactive estrogen metabolites (chemical pathway) (23, 25-27) (Figure 1). Besides these two well know mechanisms, there could be other pathways which involve extra nuclear ER such as membrane ER (mER).

![Figure 1: Schematic diagram showing the hormonal, chemical and non genomic ER signaling pathways associated with estrogen carcinogenesis.](image-url)
1.2.1. Hormonal carcinogenesis pathway

After the discovery of the correlation between estrogen and breast cancer by George Beatson, Stanly Boyd reported in 1900 that only one in three premenopausal women could anticipate disease control by oopharectomy. It was difficult to understand and explain the mechanism behind the effect of oopharectomy and breast cancer control in some women but not in all breast cancer patients, until Jenson et.al published a report about the target specificity of estrogens after about 60 years (28). Jenson and colleagues reported that the target specificity depends on the presence of estrogen receptors in target tissues which are responsible for the initiation of a cascade of biochemical events associated with estrogen action (29). Further experiments performed to understand the target specificity of estrogens led to the discovery of estrogen receptor α (ER α) for which Jenson was awarded with the Lasker award in 2004. In 1996 the discovery of another ER subtype; ER β which has a different tissue distribution compared to ER α contributed to the advancement of understanding estrogen actions (30). ER contributes to the mediation of estrogen hormonal pathway via increasing cellular proliferation in estrogen sensitive tissues.

1.2.1.1. ER and mechanism of signaling

Estrogen receptors are members of the nuclear receptor superfamily, which also includes steroid receptors, vitamin D receptors, thyroid and retinoid hormone receptors (31, 32). Although ER α and ER β are genetically encoded by two different chromosomes, they share similarities in the protein structure and function (33). Both genes that encode ERs have different tissue distribution patterns; with exclusive expression in some tissues and mixed expression in others. Expression of ER α is dominant in reproductive tissues while ER β expression is dominant in nervous system, digestive track, and ovaries (30, 34). ER α has a molecular weight of 66 kDa while ER β is 59 kDa. Estrogen receptors composed of multiple interactive functional domains; i.e. N- terminal activation function-1 (AF-1), DNA binding domain (DBD) and C- terminal ligand binding domain (LBD) and AF-2 region (33). AF-1 is involved in transcriptional activation of target genes in a ligand independent manner which is mediated via protein-
protein interactions. It is the domain that ER α and β differs most, which may also explain the different
gene activation and different ligand response with the two ER subtypes (35). The AF-1 domain of ER α
strongly activates estrogen responsive element (ERE) containing gene transcription, while ER β has little
effect on transcription of ERE containing genes (36). The DBD is the most conserved region of ER and
contains two zinc finger structures essential for receptor dimerization and DNA binding (37).

Figure 2: Schematic diagram of ER representing structural domains of ER α and ER β. DBD, DNA
binding domain; LBD. Ligand binding domain, AF-1/2: Activation function -1/2.

Few molecular mechanisms have introduced to explain the ER mediated biological functions of
estrogens. Among them the classical ligand dependent direct activation of ERE containing target genes is
the extensively studied pathway mainly involved in hormonal carcinogenesis (Figure 1). According to
this classical pathway, upon binding of a ligand, conformational changes occur in ER leading to,
dissociation of inhibitory chaperones such as Hsp70 and Hsp90 and receptor dimerization. Dimerized ER
gets translocated into the nucleus and ER binds to estrogen responsive element (ERE) in the targeted
gene. Receptor DNA complex can recruit co activators and co repressors depending on the ligand they are
bound with. Recruitment of co-activators by ER to the target gene promoter is finely controlled process
occurs in an orderly manner (38). It has been reported that the sequential addition of co-activators is
essential to achieve maximum transcription activity (39). Interaction of co-repressors with ER will inhibit the transcription of target genes. Also some co-regulators can function as both co-activators and co-repressors in a target specific manner (40).

Among the other estrogen signaling pathways studied ERE independent target gene activation by ER dimer (ER/ERE independent pathway) or tethered pathway is important. According to this pathway, upon binding of estrogen, ER α and ER β can activate gene transcription since ER can act as a transcription factor. Estradiol bound ER can either bind to DNA directly or indirectly via interacting with other response elements to activate non ERE regulated genes (41). An important example of ERE independent gene regulation by ER α is the inhibition of interleukin-6 (IL-6) gene expression mediated via NF-κB. ER α interact with c-rel subunit and prevent the binding of NF-κB to the IL-6 promoter (42).

Two other ER mediated signaling pathways have been introduced and fewer studies have done to understand the mechanism of these pathways. Ligand independent pathway which is mediated via phophorylation of ER is one of the pathways that depend on the activation of ER by extracellular signals in the absence of E₂. Ligand independent pathway induce the target gene activation through other signaling pathways such as growth factor signaling (epidermal growth factor; EGF, insulin like growth factor; IGF-1) (43). In this pathway activated kinases phosphorylate ERs and trigger dimerization, nuclear translocation, DNA binding and gene transcription regulation without direct binding of a ligand to ER (44). Although the molecular mechanism of this pathway is characterized to some extent, biological functions has to be studies further in detail to understand the involvement of this pathway in pathophysiology of cancer.

The non genomic pathway is the least studied ER signaling pathway, although it has been observed in many tissues. This pathway is believed to be mediated via membrane associated ER (mER). These mER has special characteristics that distinguished from classical ER, such as rapid response time, RNA and protein synthesis independent, involvement of mobilization of second messenger (Ca²⁺, cGMP, nitric oxide), and the frequent association of protein kinases (MAPK, PI3K/AKT) (45). Understanding the
molecular mechanisms of ER signaling is important since ER is associated with development, progression and treatment of breast cancer.

1.2.1.2. Breast cancer and ER

It has been reported that all the above mentioned ER mediated pathways are important in the development of breast cancer although some of the pathways needed to be studied further to understand the correlation to cancer (46, 47). ER mediated increased cell proliferation is the hormonal mechanism of estrogen dependent cancer formation (48, 49). ER is important in the development and progression of breast cancer. Increased cell proliferation mediated via ER resulting in an increased risk of accumulating genetic mutations during DNA replication. Estrogen related cancer formation is associated with accumulation of mutations in genes such as oncogenes, tumor suppresser genes and DNA repair genes (50).

Estrogen promotes cell growth and tumor progression by increasing the expression of many target genes such as genes associated with cell cycle, tumor metastasis, invasion (51). Genetic polymorphisms in genes involved in ER pathway showed to have an association with breast cancer risk (52). It has been reported that ER signaling pathway can regulates signal nucleotide polymorphisms (SNPs) were associated with ER tested in breast cancer patients (52).

1.3. Breast cancer prevention

Cancer chemoprevention is defined as the use of natural, synthetic or biochemical agents to reverse, suppress or prevent carcinogenesis process to neoplastic disease (53). Breast cancer remains the most commonly diagnosed malignancy in females. Studies have done to understand the underlying mechanism of breast cancer occurrence in order to find out therapeutic and preventive measures for breast cancer. Despite several factors associated with increased breast cancer risk which cannot be controlled such as age, gender, family history and genetic disposition; most of the other risk factors can be modified. Among the common risk factors that can be modified; obesity, alcohol intake, dietary habits and hormonal exposure are important and preventive measures have developed targeting these factors, mainly
hormonal exposure (54). Development of breast cancer chemopreventive agents targeted to reduce the hormonal exposure (endocrine therapy) either by reducing estrogen biosynthesis (aromatase inhibition) or attenuating the biological actions of estrogen via inhibiting the binding to ER (antiestrogens or selective estrogen modulators). Thus endocrine therapy remains the choice of treatment and prevention of ER (+) breast cancer.

1.3.1. Aromatase inhibitors (AIs) in breast cancer treatment and prevention.

Aromatase catalyze the chemical conversion of androgens to estrogens in the estrogen biosynthesis pathway. Increased levels of circulating estrogens and high aromatase expression are known to associate with increase breast cancer risk. Therefore aromatase inhibition is expected to reduce the production of estrogens and ultimately decrease the breast cancer occurrence. It has been reported that AIs increase disease free survival and benefit over tamoxifen in patients with primary ER (+) breast cancer and also reduce the risk of developing contra lateral breast cancer (55). In adjuvant therapy third generation AIs, anastrozole, exemestane and letrozole could significantly reduce the incident of contralateral breast cancer and also have shown an effect superior to tamoxifen (56). There are three major clinical studies done to understand the effects of AIs up to date, i.e. International exemestane study (IES), Arimidex tamoxifen alone or in combination (ATAC) and letrozole after 5 years of tamoxifen (MA.17). These studies have evaluated three different populations of breast cancer patients with prior exposure to tamoxifen. All three clinical trials have shown improvements in disease free survival in the individuals (56). An study done by yue et.al shown that aromatase inhibitors could inhibit both hormonal pathway and chemical carcinogenesis pathway in human breast cancer cells and the effect was superior to that of tamoxifen (57). Another study done in a nude mouse model have demonstrated that aromatase inhibitors have potent effect on mammary tumor growth, but unlike tamoxifen lack the estrogenic effect in the uterus (58). Similarly in the clinical trials done with breast cancer patients, less life threatening side effects were observed with aromatase inhibitors. There was reduced incident of endometrial cancer and thromboembolic events with aromatase inhibitors which are the major side effects with tamoxifen and raloxifene respectively (59). The major side effect associated with AIs was decreased bone density.
1.3.2. Selective Estrogen Receptor Modulators (SERMs) in breast cancer prevention

Selective estrogen receptor modulators are a group of compounds that can exert estrogenic or antiestrogenic effects in a tissue and organ specific manner (60). Mechanism of action of SERMs is described in detail in chapter 3. Tamoxifen (Tam) was the first SERM to develop in 1967 by Walpol and colleagues which showed a significant inhibition in breast cancer occurrence (61). It was reported to reduce the contralateral breast cancer by 50% when used in adjuvant therapy (62). Many clinical and preclinical studies have done to evaluate the effects of Tam in breast cancer prevention and treatment. The national surgical adjuvant breast and bowel project (NSABP) initiated the first breast cancer prevention trial (P-1) in 1993. According to the results of that study there was a 49% reduction in the development of invasive breast cancer while 50% reduction was observed for non invasive breast cancer. However this effect was only limited to ER (+) tumors while there was no effect on ER(-) tumors. This clinical trial provided important information about the mechanism of action of Tam, since it showed that ER status is crucial in the treatment of breast cancer with tamoxifen (63). In 1998 Tam achieved positive results in the breast cancer prevention trial (BCPT) leading to the approval of first cancer preventive agent by FDA (Food and Drug Administration) (64). But there was an increase in the occurrence of endometrial cancer since Tam acted as an estrogen agonist in that tissue. It was reported that in late 1980s that raloxifene (Ral); a second generation SERM, could maintain bone density in overiectomized rat and also prevent the induction of rat mammary carcinogenesis (65). Ral is in clinical use in the treatment and prevention of post menopausal osteoporosis. The STAR trial indicated that Ral also show a significant reduction in breast cancer while possessing less side effects compared to Tam (66). However there were some side effects associated with the use of Ral such as increased thromboembolic events and stroke (67). Nonetheless Ral is not the model SERM to be used as breast cancer prevention since it showed problematic drug absorption and rapid phase II metabolism which will lead to reduced bioavailability (68). There are other SERMs which have undergoing research in the prevention and treatment of breast cancer. Arzoxifene is a SERM which is in clinical use of post menopausal osteoporosis. It has been reported that arzoxifene can significantly inhibit nitrosomethylurea induced mammary carcinogenesis in
rat models (69). Also results of a phase II randomized, double blind clinical trial showed that arzoxifene is effective in the treatment of tamoxifen sensitive and tamoxifen refractory patients with advanced metastatic breast cancer with minimal toxicity (70). A study done in an animal model demonstrated that combine use of arzoxifene and rexinoid (retinoid X receptor agonist) could have preventive and therapeutic effects against breast cancer (71). It is worth considering further studies about arzoxifene in order to find out the potential to prevent and treat breast cancer since there was very little toxicities associated with that treatments (69, 71).

**1.3.3. Botanical dietary supplements in breast cancer prevention.**

Botanicals have been used in the prevention and treatment of human diseases throughout history. Botanicals classified as dietary supplements, not drugs by the Dietary Supplement Health Education Act (DSHEA) and intended to supplement the diet, contain dietary ingredients to be taken by mouth as a pill, liquid, or tablet and be labeled as a dietary supplement (72, 73). Botanical dietary supplements (BDS) by definition are not intended to diagnose or treat a disease. Nonetheless, BDS are in wide use for post menopausal symptom relief without fully understanding the safety and efficacy. Studies have shown that some of the botanicals and phytochemicals are important in the treatment and prevention of cancer. Extensive research have been done to evaluate the correlation between cruciferous vegetables, *Allium* vegetables, citrus fruits and cancer chemoprevention (74). Also phytochemicals such as resveratrol, sulforaphane and withanolides have been isolated from plants and have shown chemopreventive properties (74). It has been reported that proanthocyanidin, a phenolic compound found in fruits, vegetables, flowers, seeds and barks, can act as a chemopreventive agent by modulating multiple targets including NF-κB, mitogen activated protein kinase, PI3K/AKT, caspases, cytokines, cell cycle regulatory proteins (75). A case control study conducted in Shanghai, China showed that women with high intake of fruits and vegetables were significantly less likely to have breast cancer as compared to women with low intake (76). Another study done with premenopausal women in New York area also showed that increased intake of vegetables will reduce the breast cancer risk (77). Resveratrol is one of the phytochemicals extensively studied for its potential to act as a chemopreventive agent. Many pre clinical
and few clinical studies have done to evaluate the effect of resveratrol in the prevention and treatment of cancer (78, 79). It has been shown that resveratrol can affect all three stages of carcinogenesis (initiation, promotion, progression) by modulating signaling pathways associated with cell cycle, apoptosis, inflammation, angiogenesis and metastasis (80). Further studies are needed to understand the safety, efficacy and the mechanism of action of botanicals and phytochemicals in the prevention and treatment of cancer.

1.3.4. NO/cGMP and other signaling pathways in the prevention of breast cancer.

Nitric oxide is a free radical secondary messenger molecule which is important in inter and intra cellular signal transduction (81). Biosynthesized of NO from L-arginin is catalyzed by nitric oxide synthase (NOS). NO can act as a vasodilator, a modulator of neurotransmission and a defense against pathogens (81). However there has been studies done to understand the potential role of NO in cancer as well (82). NO can be either useful or harmful depending on it chemical fate, rate and location of production in mammalian cells (83). It has been reported that NO pathway is involved in tumor angiogenesis and metastasis (84). Increased production of NO could select mutant p53 cells and could contribute to tumor angiogenesis by up regulating vascular endothelial growth factor (VEGF) (85). It was reported that NO biosynthesis was significantly greater in grade III breast tumors compared to grade II tumors, suggesting that increased NO production could contribute to tumor progression (86). Also a study done by Nakamura et. al showed that NO could stimulate VEGF-C expression in breast cancer cells and increase the lymph node metastasis (87). Contradictory results regarding NO have been published showing that increased NOS expression may be cytotoxic for tumor cells while low level activity can promote tumor growth (85). However modulation of NO/cGMP pathway can be of great interest regarding cancer prevention and treatment since NO pathway is involved in many important pathophysiological processes.

Among many signaling pathways involved in tumor initiation, progression and metastasis; NF-κB pathway is extensively studies for its importance in carcinogenesis. NF-κB is involved in the regulation of
wide variety of biological responses. It regulates the expression of many genes involved in carcinogenesis including the genes involved in proliferation, migration and apoptosis (88). A study done in human breast epithelial cells (MCF-10A) have shown that 4-OHE$_2$ induced malignant transformation of these cells is achieved by induction of NF-κB expression (89). It has been reported that there is a positive crosstalk between ER and NF-κB in breast cancer cells, and they can act together to promote cancer cell survival and increase the progression to a more aggressive phenotype (90). Results from preclinical studies suggested that inhibition of NF-κB in endocrine resistant, ER (+) breast cancer cells could restore their sensitivity to standard endocrine therapy such as Tam (91). It was reported that genistein, a soy isoflavone could inhibit NF-κB pathway thereby induce apoptosis in prostate cancer cells (92).

Many other signal transduction pathways are involved in the initiation, progression and metastasis of cancer including but not limited to, MAPK/Akt pathway, Wnt/β-catenin signaling and NRF2/keap1. Modulation of these pathways possibly provides a potential to find therapeutic and preventive agents against cancer.

1.4. Specific aims for the study

Estrogen dependent breast cancer development is due to long term exposure to estrogens via a combination of early onset of menstruation, delayed first child birth, short duration of breast feeding, late menopause, and use of hormone replacement therapy (HRT) (93). The goal of this study was to discover effective botanical dietary supplements, SERMs and NO modulators for breast cancer chemoprevention in an in vitro system. It is hypothesized that botanicals, SERMs and NO/cGMP pathway modulators could attenuate oxidative estrogen metabolism and estrogen induced malignant transformation in human breast epithelial cells and could act as breast cancer chemopreventive agents. Major focus of this project was to determine the effects of botanicals, SERMs and NO modulators on oxidative estrogen metabolism in vitro and to explore the effects on oxidative stress and estrogen induced malignant transformation in human breast epithelial cells.

In order to test the above hypothesis, three specific aims were designed as indicated below.
I. Evaluation the effects of botanical dietary supplements on estrone methyl ether formation and E$_2$ induced malignant transformation in MCF-10A cells.

II. Evaluation the effects of SERMs on estrone methyl ether formation, oxidative stress, E$_2$ induced ROS formation and malignant transformation in MCF-10A cells.

III. Evaluation the effects of NO/cGMP pathway modulation on estrogen metabolism, E$_2$ induced ROS formation and malignant transformation in MCF-10A cells.
2: MECHANISM AND BIOMARKERS OF ESTROGEN CHEMICAL CARCINOGENESIS

2.1. Introduction

2.1.1. Mechanism of estrogen chemical carcinogenesis.

Long term exposure to endogenous estrogens either through early menarche or late menopause will increase the risk of developing hormone dependent cancer in women (94). One of the mechanisms that could contribute to estrogen carcinogenesis is the chemical carcinogenesis mechanism which can occur due to the imbalance of estrogen metabolism (95). Biosynthesis of estrogens in pre-menopausal women mainly takes place in the ovaries and placenta (during pregnancy), while in postmenopausal females adipose tissue and adrenal gland serve as the major sites for estrogen synthesis (96). Cholesterol act as the precursor for the biosynthesis of androstenedione and aromatase catalyzes conversion to estrone (E₁). Also in testosterone synthesis, cholesterol acts as the precursor and aromatase convert testosterone to estradiol (E₂). E₁ can be converted to E₂ by 17 β- hydroxysteroid dehydrogenase (17β-HSD). Both E₂ and E₁ are metabolized giving a similar metabolic profile. In breast epithelial cells both E₂ and E₁ are oxidized to 4- and 2-hydroxycatechols in the presence of CYP450 1B1 and 1A1, respectively (95) (Figure 3). Both catechols are further oxidized to form reactive quinones and 3,4-OHE₂/E₁-o- quinone is considered more genotoxic (97). A study done by Fernandez et.al, showed that E₂, 4-OHE₂, and 2-OHE₂ can induce mutations in MCF-10F cells although the concentration needed to induce mutations is higher for 2-OHE₂ compared to E₂ and 4-OHE₂ (98). The genotoxicity of quinones could be due to their electrophilic reactivity which will lead to the formation of DNA and protein adducts under physiological conditions. It has been shown that DNA single strand damages were mediated by 3, 4-estrone quinone in MCF-7 human breast cancer cells (99, 100). Also depurinating DNA adduct formation has been reported by estrogen quinones in breast epithelial cells (101).

In addition to the toxicity caused by highly electrophilic estrogen o-quinones generation of reactive oxygen species (ROS) could also cause genotoxicity. During the oxidation of catechols to quinones, generation of ROS can occur due to redox cycling and eventually cause genotoxicity (Figure...
Elevated levels of ROS can cause harmful effects via oxidation of DNA, lipids, proteins, and enzyme co-factors. A study done using different breast cancer cell lines demonstrated that E₂ could rapidly produce intracellular ROS independent of the ER status (102). Oxidative DNA damage can be implicated of cancer risk and ROS can serve as a useful index of oxidative stress. Furthermore, oxidized derivative of deoxyguanosine, 8-oxo-2'-deoxyguanosine (8-oxo-dG) serve as a useful biomarker in oxidative DNA damage in vivo and in vitro (103).

Phase II conjugative and detoxification reactions will reduce the toxicity of catechol estrogens. In breast epithelium, catechol estrogens (CE) are conjugated with sulfate groups, glucuronic acid and/or methyl groups in the presence of sulfotransferase (SULT), uridine 5’-diphospho-glucuronosyltransferase (UGT), and catechol-o-methyl transferase (COMT), respectively. Although COMT can use most of the catechols as substrates, CEs have the highest affinity towards this enzyme (104). Methylation is the most widely studied detoxification pathway with regards to CEs, while detoxification of CEs by sulfation and glucuronidation remain least explored areas so far. SULTs catalyze the transfer of a sulfonyl group (SO₃⁻) using 3’-phosphoadenosine-5’-phosphosulfate (PAPS) as the sulfonate donor (105). While both CEs and parent estrogens can get sulfated and glucuronidated, CEs are detoxified mainly via methylation (104).

Figure 3: Oxidative estrogen metabolism and genotoxicity in breast epithelial cells
Detoxification of highly reactive catechol estrogen quinones (CE-Q) can occur by the formation of GSH conjugates which is catalyzed by glutathione S-transferase (GST) (106). GSH conjugation to occurs both in vivo and in vitro and GSH conjugates are converted to mercapturic acids and rapidly excreted from the body (104). It has been reported that even after GSH conjugation, CE-Qs can undergo redox cycling producing ROS and creating oxidative stress (106). Yet there is evidence that a net reduction of DNA damage was observed with GSH conjugation (107).

Imbalanced estrogen metabolism is associated with estrogen carcinogenesis in breast epithelium (108). There is a growing body of evidence which shows that increased production of catechol and quinones and reduced levels of phase II conjugation is associated with increased risk of estrogen carcinogenesis. A study done with primary breast cancer patients showed that the levels of E2 and E1 are elevated in breast tissue compared to urine (109). Evidence is limited regarding the correlation between estrogen metabolism and breast cancer risk analysis in healthy individuals. A study done by Fuhrman and colleagues showed that a lower risk of breast cancer occurrence in postmenopausal women is associated with increased levels of 2-hydroxylation and a higher risk is associated with less extensive methylation of 4-hydroxy catechols (110). A case control study done with premenopausal women showed that increased levels of parent estrogens in urine was correlated with lower risk, but mid-luteal urinary samples did not show a significant pattern in the 2 and 4-hydroxycatechols (111). The mechanism of estrogen chemical carcinogenesis depends on the balance of estrogen metabolism. Impaired metabolism results in increased production of CEs and reduced phase II conjugates will leads to genotoxicity and carcinogenesis.

2.1.2. Role of estrogen metabolizing enzymes in chemical carcinogenesis

2.1.2.1. Cytochrome P450 enzymes (CYP450)

CYP450 enzymes are the major drug metabolizing enzymes in human body and responsible for about 75% of drug metabolism (112). CYP enzymes are a super family of heme containing monooxygenases that are involved in oxidative metabolism of many compounds including steroids (113). Cytochrome P450 enzymes are highly expressed in liver and metabolism of estrogens take place mainly in the liver producing approximately 80% of 2-hydroxycatechols and 20% of 4-hydroxycatechols (114).
Oxidation of estrogens to 2-hydroxycatechol is mainly mediated via CYP450 1A2 and CYP450 3A in the liver and via CYP450 1A1 in the breast and endometrium (114). CYP450 1B1 shows high reactivity toward polycyclic aromatic hydrocarbons such as benzo[a]pyrene and also highly specific for the catalysis of 4-hydroxylation of E2 (115, 116). Since CYP450 1B1 is abundantly expressed in estrogen sensitive tissues such as breast and uterus, 4-hydroxylation of estrogens is prominent in these tissues (117, 118). After identifying the complete cDNA sequence for CYP450 isoform 1B1 and isolating and characterizing the CYP1B1 gene, extensive research has been done to understand the importance of CYP450 1B1 in pathophysiology of many diseases (119, 120). There is evidence showing that there is a change in the expression levels of CYP450 enzymes in normal breast epithelial cells and breast cancer cells (121). It has been reported that higher amount of CYP450 1B1 is localized into breast cancer cells assessed by immunohistochemistry (122). But a contradictory report was published by Iscan et. al showing that there is no quantifiable difference in the mRNA levels of CYP450 enzymes in breast cancer cells and surrounded normal cells in the same tissue sample as analyzed by RT-PCT (123). There are studies to show that an environmental toxin, (2,3,7,8-tetrachlorodibenzo-p-dioxin) TCDD can induce CYP450 1B1 and 1A1 in breast and endometrial cells to produce more catechols upon exposure to E2 (124-127). Several studies have shown that the mechanism of induction and/or suppression of CYP450 1B1 and 1A1 expression is ER mediated and depends on ER status (121, 128, 129). However, Deb et. al showed that E2 mediated suppression of CYP1B1 expression in MA-10 cells (mouse Lydig cells) is ER independent (130). There are evidence showing that CYP450 1B1 expression modulation is via the aryl hydrocarbon (AH) receptor (131, 132). Tsuchiya and colleagues showed that the regulation of expression of human CYP450 1B1 is under the control of microRNA as well (133). There is evidence showing that polymorphisms in CYP450 3A4 and 1B1 are associated with prostate cancer (134). However, widely studied single nucleotide polymorphisms of CYP1B1 are not associated with breast cancer risk (135), whereas polymorphisms in CYP1A1 are associated with breast cancer risk in Caucasian women (136). Due to the importance of CYP450 1B1 in estrogen metabolism and carcinogenesis, it has been a good target in the development of anticancer therapeutic strategies. Several compounds has been discovered to
inhibit the CYP450 1B1 activity (acetylenes, α-napthoflavone, resveratrol) (137-139) and to suppress the CYP450 1B1 expression (resveratrol) (127) which can be used as potential anticancer therapeutic compounds.

2.1.2.2. Phase II conjugative metabolism

Phase II detoxification and conjugative metabolism of estrogens is essential as it will reduce the reactivity of catechols and quinones. Sulfation and glucuronidation of parent estrogens; methylation, sulfation and glucuronidation of catechols, and glutathionylation of quinones are the main phase II detoxification reactions take place with regard to estrogen metabolism (104). Apart from above reactions, conversion of quinones to catechols by NQO1 is also considered as a detoxification reaction.

Sulfotransferases (SULTs) are a subfamily of transferases which catalyze the transfer of a sulfate group from the ubiquitous donor 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to an acceptor molecule (140). Although liver is the major site for estrogen sulfation, it can occur in extrahepatic tissues such as intestine, breast, and brain to a lesser extent. SULTs responsible for steroid sulfation are found in the cell cytosol and the conjugation of sulfate groups to estrogens is mainly mediated by sulfotransferase 1E1 (SULT 1E1) (140). It has been reported that SULT 1A1, 1A2, 1A3, 1C4 and 1E1 can use catechol estrogens as substrates while 1E1 possessing the strongest sulfating activity towards catechol estrogens (141, 142). There is a balance in the rate of sulfation by SULTs and the rate of removal of sulfate group by sulfatase. It has been reported that during carcinogenesis this balance will be interrupted producing less sulfated estrogens and more free estrogens (143, 144). SULT1E1 expression was reduced while sulfatase expression was induced in breast cancer tissue samples suggesting that during carcinogenesis sulfate group conjugation can be modulated in such a way to produce more free E2 (145).

Uridine 5'-diphospho-glucuronosyltransferase (UGT) is a member of glycotransferase enzyme family which catalyze the glucuronidation reaction to produce a β-D-glucuronide product (146). Conjugation of a glucuronic acid group will generally result a less biologically active product with increased water solubility and acidity to facilitate the excretion via bile or urine (147). Several UGT isoforms are important in the estrogen phase II conjugation, which mainly take place in the liver. UGT
1A1 is the extensively studied UGT enzyme which can use different lipophilic compounds such as estrogens, other steroid hormones and bilirubin as substrates (148). Mutations in liver UGT 1A1 is associated with many diseases related to bilirubin metabolism, such as Gilbert syndrome and Crigler-Najja Syndrome. Although a high expression of UGT enzymes can be seen in the digestive system (mainly in liver and biliary tissue); different UGT isoforms were expressed in many vital organs as well.

UGT1A9, UGT2B4, UGT2B10, UGT2B11, UGT2B15 and UGT2B17 are among the UGT enzymes predominant in the breast (148). It has been reported that a higher catalytic activity shown by UGT 1A1 and 1A3 towards 2-hydroxyestrogen glucuronidation while UGT 2B7 showed a higher activity towards 4-hydroxyestrogens (149). In ER (+) breast cancer cells, E2 up regulate the expression of UGT2B15, which is the only known UGT2B enzyme up-regulated by estrogens; in an ER dependent mechanism (150).

Genetic polymorphism of UGT is associated with cancer risk. It has been reported that polymorphism in UGT1A1 gene leading to low catalytic activity is associated with invasive breast cancer risk in women with African American origin (151). Another study done with Chinese women showed that a polymorphism in *28 allele in the UGT1A1 gene leading to reduced activity of UGT1A1 enzyme, predisposed the individuals to an increased risk of breast cancer (152). Changes in the expression and activity of UGT enzymes associated with cancer risk as well. Expressions of UGT 2B7, 2B10 and 2B15, which are normally expressed in melanocytes; were suppressed in melanoma, suggesting that malignant transformation affect the UGT expression (153). It has been reported that higher mammographic density, which is positively associated with the risk of developing breast cancer (154); is associated with lower expression of UGT. Also in the same study it was reported that a lower expression of UGT was seen in biopsies from newly diagnosed breast cancers (155). It has been reported that the modulation of UGT enzymes is mediated via ER, AHR, CAR and other orphan receptors, and/or Nrf2/keap 1 pathway; yet the molecular mechanism is still not well understood (131, 150, 156, 157).

**NAD(P)H–quinone oxidoreductase (NQO1)** catalyzes 2 electron reduction reactions and is responsible for the conversion of quinones to hydroquinones. Although NQO1 is considered as a detoxification enzyme in estrogen metabolism, it increases the production of catechols promoting redox
cycling and ROS formation eventually. It has been reported that both Nrf2 and NQO1 are suppressed in E_{2} exposed mammary tissue and in mammary tumors in ACI rats after treating with E_{2} for 240 days and antioxidants could reverse the expression levels (158). NQO1 can act as an antioxidant via generation of antioxidant forms of ubiquinone and vitamin E (159). A study done with human breast tumor samples showed that there is an inverse correlation of NQO1 and NF-κB yet neither of them cannot be used as prognostic or predictive markers in breast cancer(160). NQO1 is inducible and its gene transcription is modulated and controlled mainly via Nrf2-Keap1 pathway (161, 162). Due to the inducibility of NQO1 upon exposure to antioxidants, measurement of induction of NQO1 activity is used as a screening assay for the analysis of potential chemopreventive and anti-carcinogenic compounds (163, 164). Induction of phase II/conjugative enzymes can be a better way to discover anticancer/chemopreventive drugs(165).

2.1.3. Model cellular system to study estrogen chemical carcinogenesis

In order to understand the modulation of estrogen chemical carcinogenesis, a model system has to be selected in such a way that the influence from hormonal pathway is minimal. MCF-10 cells are spontaneously immortalized, non tumorigenic, ER (-) human mammary epithelial cells. Two sublines of MCF-10 cells have been isolated and characterized, viz, adherent cells, MCF-10A and floating cells MCF-10F. MCF-10 cells are considered having normal breast epithelial like properties since they possess several characteristics equivalent to normal breast epithelial cells, such as (a) lack of tumorigenicity in nude mice, (b) three dimensional growth in collagen, (c) growth in culture that is controlled by hormones and growth factors, (d) lack of anchorage independent growth and (e) dome formation in confluent cultures (166). Previous studies have shown that MCF-10 cells can be transformed into a malignant phenotype upon exposure to estrogens and estrogen metabolites (167-169) and form anchorage independent colonies in soft agar. MCF-10 cells have been previously used in the studies related to estrogen metabolism, reactive oxygen species formation and estrogen induced DNA adduct formation suggesting that this could be a good in vitro model system to study about estrogen chemical carcinogenesis (89, 126). In the present study MCF-10A cells were used as the cellular system to study estrogen chemical carcinogenesis since the influence from hormonal pathway is minimal. In addition,
main phase I enzymes responsible for extra hepatic estrogen metabolism, which are CYP450 1A1 and 1B1 are expressed in this MCF-10 cells, making it a better system to used in the present study.

2.1.4. Biomarkers in estrogen carcinogenesis

Since breast cancer is the second leading cause of cancer deaths among American women, early detection can be of great help to minimize the metastasis, morbidity, and mortality due to breast cancer. Mammography is the widely used method to identify and predict the occurrence of breast cancer. Early detection of breast cancer risk will be of great use if it is more specific, non invasive and selective biomarker for estrogen carcinogenicity. Existing breast cancer biomarkers provide understanding in early diagnosis, prognosis, response to therapy, and aid in surveillance after primary therapy (170). Although, tumor markers such as carcinoembryonic antigen (CA) 15-3, 27.29 are used to monitor disease progression and recurrence, none of the existing tumor markers are sensitive enough to detect low volume disease and specific enough to correlate with the tumor status making it difficult to use as an early detection tool (171). Among the well established biomarkers in the prediction and treatment assessment, ER status is important, since more than 80% of invasive breast cancers are ER (+). In addition to testing the ER status, progesterone receptor and the oncogene HER2 status are two another widely used biomarkers in breast cancer (172). Other potential biomarkers are desperately needed which can be used in the early detection of breast cancer.

Detection and quantification of estrogen metabolites in tissue and in urine samples can be used as a sensitive and specific biomarker in the early detection of estrogen carcinogenesis. There is evidence showing that during the development of breast cancer, the estrogen metabolism profile will change in such a way to increase the oxidative metabolites and to reduce phase II conjugation (173-176). Therefore estrogen metabolism could be used in for breast cancer risk prediction (177-179). Detection of 2-hydroxyestrogens in human urine samples have been used as a good breast cancer risk predictor and there are evidence showing that there is an higher incidence of ER (+) breast cancer occurrence in the women taking HRT and with higher levels of circulating 2-hydroxyestrogens (180). In previous studies, 2/16 hydroxyestrogen metabolite formation was used in the prediction of breast cancer risk in post and pre
menopausal women (110, 111). A metabolism study done by Im and colleagues showed that a lower urinary 2:16 OHE ratio is associated with high risk of breast cancer and the ratio for breast cancer patients and high risk women was within the same range (181).

Highly reactive estrogen quinones can react with DNA to form depurinating adducts, which can be used as potential biomarkers in breast cancer. Among the 2 estrogen catechols, 4-hydroxyestradiol is considered more genotoxic since the 4-hydroxyestrogen quinine can form depurinating DNA adducts more rapidly. Measurement of serum and urinary DNA adduct has been used in previous studies to predict the disease progression and elevated serum estrogen-DNA adducts are considered as potential biomarkers in breast cancer risk prediction (182, 183). It is not known whether the available methods are sensitive enough and can get a reliable measurements of DNA depurinating adduct formation as biomarkers to oxidative estrogen metabolism and breast cancer risk prediction, since the same DNA adducts can be formed from the nucleotide pool of the cells (184).

It has been reported that there is higher amount of ROS formation and reduced activity of catalase in the breast cancer, suggesting that oxidative stress could be a possible biomarker in breast cancer (185). ROS is produced in the cellular reactions and when the production of ROS is exceeded the scavenging capacity of the enzymes, increased DNA damage can be resulted. One of the widely studied DNA oxidative product 8-oxo-dG generation is measured and quantified as an indication of oxidative stress and reported to be a potential independent prognostic factor in breast cancer (186, 187). It has been reported that estrogen and estrogen metabolites can induce oxidative stress (188) leading to the development and progression of breast cancer (189). All of these reports support the fact that ROS and 8-oxo-dG can be used as biomarkers of oxidative stress and carcinogenesis.

In the present study, measurement of estrogen metabolites in cultured human breast epithelial cell media was used to predict the effect of different compounds in the estrogen carcinogenicity. Since the effect on COMT was minimal from all the treatments used in the study, formation of methyl ether metabolites were measured assuming that methyl ether formation is reflective of the catechol formation. This method can be further used to detect and predict carcinogenicity and tumorigenicity in animal
models and human samples. Present study was conducted to understand the mechanism of estrogen chemical carcinogenesis in human breast epithelial cells and to find possible biomarkers in estrogen carcinogenesis.
2.2. Materials and methods

2.2.1. Cell culture conditions

MCF-10A, human breast epithelia cells were obtained from American Type Cell Collection (Manassas, VA) and maintained in Dulbecco’s modified Eagle’s medium and F12 medium (DMEM/F12) supplemented with 1% penicillin-streptomycin, 5% fetal bovine serum, cholera toxin (0.1 µg/mL), epidermal growth factor (20 ng/mL), hydrocortisone (0.5 µg/mL), insulin (10 µg/L) and 5% CO₂ at 37 °C as described previously (190). Estrogen-free medium for MCF-10A cells were prepared supplementing charcoal-dextran treated fetal bovine serum to phenol red free DMEM/F12, whereas other components remain the same.

2.2.2. Chemicals and reagents

All the chemicals and reagents were obtained from Sigma (St. Louis, MO) or Invitrogen (Carlsbad, CA) unless stated otherwise. Antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), Cell Signaling technology (Boston, MA) and Sigma (St. Louis, MO). Reagents used for SiRNA experiments were ordered from Santa Cruz Biotechnology (Santa Cruz, CA). All the standard compounds of estrogen metabolites were obtained from Steraloids Inc. (Newport, RI). Deuterated estrogen metabolites were obtained from CDN isotope (Pointe-Claire, Quebec).

2.2.3. Analysis of estrogen metabolites in MCF-10A cells

MCF-10A cells were plated in 6- well plates at a density of 20,000 cells per well in estrogen free media. Cells were allowed to attach to the plate overnight and incubated with E₂ (1 μM) for 6 days. Treatments were renewed every 3 days. Cell media were collected (5 mL/well) after 3 days of treatment and stored at -20 °C after adding 2 mM of ascorbic acid. At the end of 6 days of treatment, cell media were collected and pooled with the third day cell media resulting 10 mL final volume. Ascorbic acid (2 mM) and 5 nM of internal standards were added into each sample before processing. Estrogen metabolites were extracted to dichloromethane. Dichloromethane was evaporated under a stream of nitrogen gas and reconstituted with 200 μL of 0.1 M sodium bicarbonate buffer (pH = 9) and 200 μL of freshly prepared
dansyl chloride (1 mg/mL in acetone). The reaction mixture was incubated at 60 °C for 10 min to complete the derivatization. Samples were analysed for estrogen metabolites using LC/MS-MS.

2.2.4. Analysis of estrogen metabolites using LC/MS-MS

The analytical method previously described by Xu et. al. (191) was adopted with some modifications for the detection and measurement of estrogen metabolites. All the metabolism experiments were performed using positive ion electrospray tandem mass spectrometric methodology on a API 3000 (Applied Biosystem, Forster City, CA) triple quadruple mass spectrometer equipped with Agilent 1200 HPLC (Agilent Technologies, Santa Clara, CA). Liquid chromatography was performed on a 150 mm × 3 mm i.d. column packed with 3.5 µm particles, XBridge C-18 column (Waters, Milford, MA). The mobile phase, operating at the flow rate of 300 µL/min consisted of water with 0.1% (v/v) formic acid as solvent A and 0.1% (v/v) formic acid in methanol as solvent B. Initial conditions for the 30 min run were set at 80% solvent B. The chromatographic gradient was held at the initial conditions for 5 min followed by a linear gradient of B from 80% to 95% over 20 min and held at 95% solvent B for 5 min. The mass spectrometer parameters were optimized as follows: the ionspray voltage was 4.5 kV, the source temperature was 350 °C, the nebulizer gas was 12 instrument units, the curtain gas was 8 units, and the collision gas was 5 units. The focusing potential (FP) was 370 and the declustering potential (DP) was 81 V. The collision energy for 2-MeOE₁, 4-MeOE₁, and 2-MeOE₁-d₄ was 59 V while for 2-OHE₁, 4-OHE₁ and 4-OHE₁-d₄ it was 51 V. Collision energy for E₂, E₁ and E₁-d₄ was 57 V. Multiple reactions monitoring (MRM) channel of 504 → 171 was set to detect E₁ while 506 → 171 was set to detect E₂. MRM channel of 534 → 171 was set to detect both 4-MeOE₁ and 2-MeOE₁, while 757 → 170 and 538 → 171 were set to detect 4-OHE₁-d₄ and 2-MeOE₁-d₄, respectively. Deuterated estrogen metabolites were used as the internal standards and added soon after cell media were collected for processing. Estrogen metabolites were analyzed using analyst software (Applied Biosystems). Peak areas of 2 and 4-MeOE₁ were normalized against 2-MeOE₁-d₄ internal standard to obtain the relative peak areas. 2 and 4-MeOE₁
relative peak areas in E₂ treated sample were considered as 100% and all the other samples were normalized against that and represented as percentage MeOE₁ formation.

2.2.5. Enzyme expression analysis with western blot

Protein lysates were prepared and electrophoresis was done with SDS-PAGE to separate proteins. Each lane was loaded with 30 µg of total protein as determined by BCA assay. Proteins were transferred to PVDF membrane and blocked with the blocking solution (5% non fat milk in TBS with 0.1% tween 20) to minimize non specific protein binding. Anti-CYP450 1B1, anti-CYP450 1A1, anti-SULT1, anti-SULT1E1, anti-NQO1 and anti-β-actin antibodies were used as primary antibodies in 1:200, 1:1000, 1:1000, 1:1000, 1:500 and 1:2000 dilutions, respectively. Antibodies were diluted in blocking solution. Blots were incubated with primary antibody overnight at 4 °C and with secondary antibody for 1 h at room temperature. Blots were visualized using chemiluminescence substrate (Thermo scientific, Rockford, IL). β-Actin level was measured as a gel loading and transferring control. Imaging and analysis was done using FluroChem software (Cell Biosciences, Santa Clara, CA). When quantifying proteins, each protein band density was normalized to the respective β-actin band density and was represented as the relative protein expression. Three independent experiments were done to get the average and the results were represented as average ± SD.

2.2.6. Estrogen/ estrogen metabolites and malignant transformation

Malignant transformation was analyzed using anchorage independent colony formation assay as previously described (192) with minor modifications. Briefly, MCF-10A cells were plated in T-75 flasks at a density of 0.5 x 10⁶ cells per flask and treated with E₂ 1 µM and DMSO for three weeks. In order to optimize the conditions for the anchorage independent colony formation assay, formation of colonies in agar was analyzed in a time dependent manner. DMSO (0.01%) was used as the vehicle control while Ras oncogene mutated MCF-10A cells were used as positive control, as they showed a malignant phenotype. Since there was a significant increase in the colony formation with E₂ (1 µM ) compared to DMSO after 3 weeks on agar; 3 weeks were used as the optimum time point for all the other experiments. Therefore for
the analysis of malignant transformation, cells were treated with the compounds for 3 weeks and plated on agar for another 3 weeks before counting the colonies (190). Treatments were done with E$_2$ (1 µM and 5µM), 4-OHE$_2$ (1 µM and 5 µM) for 3 weeks, twice a week. Cells were passaged once a week before it was fully confluent. At the end of the treatment, cells were seeded on soft agar (0.3% agar) at a density of 5 x 10$^4$ cells/well in 12 well plates pre-coated with 0.6% agar base medium. Estrogen free media was added as the feeding media on top of the soft agar layer. Cells were maintained in soft agar for 3 weeks and media was refreshed every 3 days. After 3 weeks, colonies were stained with crystal violet (0.05%) and analyzed using an Olympus inverted microscope (Center Valley, PA). Spherical formation of > 50 cells were taken as a colony. All the experiments were done in at least triplicates. Average number of colonies formed in one well of the DMSO treated sample was taken as 1. Average number of foci formed in other samples were normalized to the DMSO control and represented as colony formation efficacy ± SD and graphed against the treatment compound.

2.2.7. Detection of reactive oxygen species formation in live cells

MCF-10A cells were grown (4 x 10$^3$ cells/mL) on each of eight chambers on a sterile NuncTM chambered coverglass and incubated overnight at 37 °C with 5% CO$_2$ in estrogen free media and formation of ROS was determined as previously described (193) with minor modifications. Cells were treated with E$_2$ (1 µM) and DMSO for 6 days. Treatments were renewed after 3 days. At the end of 6 days media was removed and cells were labeled with CM-H$_2$DCFDA (10 µM) for 30 min at 37 °C, 5% CO$_2$. Cells were rinsed with PBS to remove excess dye and nuclear was stained with 0.2 µg/mL Hoechst stain. Imaging of the cells was performed using Zeiss 510 META laser scanning confocal microscope.

2.2.8. Estrogen related gene knock down with siRNA

Genes were knocked down with siRNA and followed the methods in the manufacturer manual protocol. Briefly, MCF-10A cells were plated in 6- well plates at a density of 2 x 10$^5$ cells per well in 2 mL of antibiotic free normal growth media with FBS. Cells were incubated at 37 °C until the cells were 80% confluent. siRNA (3 µL) for CYP 1B1 was mixed with 100 µL of transfection medium (solution A)
and 3 μL of siRNA transfection reagent was mixed with 100 μL of transfection medium (solution B). Solution A was mixed well with solution B and incubated for 30 min at room temperature. Cells were washed once with 2 mL of siRNA transfection medium and aspirate completely. Transfection medium (0.8 mL) was mixed well with solution A + solution B and total of 1 mL of that master mix was added into each well. Cells were incubated for 6 h at 37 °C in a CO₂ incubator. After 6 h normal growth media containing serum was added into each well and the plates were incubated at 37 °C for 24 h. Cell media was aspirated and cells were kept in normal growth media with stripped FBS. Cells were then treated with E₂ (1 μM) for 3 days and cell media was analyzed for estrogen metabolites using LC/MS-MS. Two independent experiments were done in duplicates to get the average values for metabolites and the p values were calculated with ANOVA.

2.2.9. Receptor translocation with estrogen and estrogen metabolites

MCF-10A cells were grown (4 ×10³ cells/mL) on each of eight chambers on a sterile NuncTM chambered glass slide and incubated overnight at 37 °C with 5% CO₂ in estrogen free media and cells were treated with 3- methylcholanthrene (3-MC) (1 μM) E₂ (1 μM), 4-OHE₂ (1 μM) and DMSO (0.01%) for 24 h. Media was aspirated and cells were washed with PBS twice. Cells were fixed by adding 10% formaline for 30 min. Then cells were washed twice with PBS and increase the permeability by adding triton X-100 (0.5% by volume in PBS) for 10 min. Cells were washed with PBS twice and 1% BSA (1mg of BSA in 100 mL water = 1% BSA) was added and incubated at room temperature for 20 min. Anti-AHR antibody (Sigma, St. Louis, MO) was used as the primary antibody and was diluted (2 μg/mL) in 1% BSA prior to adding into cells and incubated at room temperature for 1 h. Cells were washed with PBS twice and Alexa fluor 488 goat anti-rabbit IgG was used as the secondary antibody (1:500 dilution) and incubated for 30-45 min at room temperature. Cells were washed with PBS and antifade/ DAPI was added and slides were sealed for imaging. Cells were imaged using Zeiss 510 META laser scanning confocal microscope.
2.3. Results

2.3.1. Estrogen metabolite profile in MCF-10A cells

Dansyl derivative of estrogen metabolites were detected using API3000 mass spectrometry. Dansyl derivatives of estrogen metabolites were detected as the parent ions and dansyl group was detected as the daughter ion after ionization. When MCF-10A cells were incubated with E2 for 6 days, the amount of estrone (E1) detected in the media was higher than the amount of E2. Also there was an increased amount of estrone metabolites present compared to E2 metabolites. Catechol metabolites of estrone (2-OHE1 and 4-OHE1) and methyl ether metabolites (2-MeOE1 and 4-MeOE1) of estrone were detected and the amount of methyl ether formation was higher than that of catechol formation. Also higher amount of 2-MeOE1 was observed compared to 4-MeOE1 in the E2 treated samples. Methyl ether metabolites and catechols of E2 were detected in E2 treated samples and the amount of catechol formation was very low compared to methyl ether formation (Figure 4). However, consistent detection of E2 catechols in cell media was difficult, perhaps due to the instability of the catechols. There was one additional peak that consistently appeared in the 536 → 171 panel which could be due to 3-MeOE2. The detection of catechols was not consistent and the measurements were not reliable due to reduced stability of these compounds in biological samples. Therefore amount of estrone methyl ether was measures as biomarkers of estrogen metabolism in E2 and E2+compounds treated samples.
Figure 4: Representative chromatograms of E₁ and E₂ metabolites which were analyzed using the LC/MS-MS. Peaks corresponding to (A) E₁, (B) E₂, (C) MeOE₁, (D) MeOE₂, (E) OHE₁ and (F) OHE₂ were detected in a single LC/MS-MS run. All the chromatograms are represented in extracted ion monitoring (XIM) mode. MCF-10 A cells were treated with E₂ (1 µM) for 6 days. Cell media was collected and the metabolites were extracted in dichloromethane followed by derivatization using dansyl chloride. Samples were analyzed by LC/MS-MS in multiple reaction monitoring (MRM) mode.
2.3.2. Effect of E2 on metabolizing enzymes.

Protein lysates were tested for the expression of phase I and phase II detoxification enzymes after treating MCF-10A cells with E2 for 6 days. There was an increase in the expression of CYP 450 1B1 and 1A1 in a time dependent manner (Figure 5). Expression induction of CYP450 1A1 was more rapid compared to CYP450 1B1. The difference in expression induction of CYP450 1B1 and 1A1 with E2 could be possibly due to the higher inducibility of CYP 1A1 gene compared to CYP 1B1. There was a significant induction in CYP450 1B1 in day 6 compared to day 1 (p < 0.005), but the expression induction of CYP450 1A1 was not significant compared to day 1 and day 6 (p = 0.052) determined by ANOVA.

Figure 5: Expression of CYP450 1B1 and 1A1 was induced with the treatment of E2 (1 μM) in a time dependent manner. MCF-10A cells were incubated with E2 (1 μM) for 6 days and protein lysates were analyzed with western blotting. Each lane was loaded with 30 μg of total protein. Expression of CYP450
Expression of SULT1E1 and 1A1 was not significantly affected by E₂ (1 μM) treatment evaluated tested by western blot (Figure 6). Among the detoxification enzymes tested there was an inhibition in the expression of NQO1 with E₂ treatment compared to the control treated with DMSO. The amount of UGT1A1 present in MCF-10A cells was very low and it was below the level of detection of UGT1A1 antibody in western blot. But the gene expression of UGT1A1 could measure by real time quantitative PCR (qPCR) and there was an increase in the UGT1A1 gene transcription in E₂ treated samples compared to the DMSO treated controls (Figure 6). Although there was no significant increased in the SULT1E1 and 1A1 protein levels with E₂ treatment tested by western blot, there was an increase in the mRNA levels of both genes with the treatment of E₂ (1 μM) assessed by qPCR (Figure 6).
Figure 6: (A). Expression of phase II detoxification enzymes were analyzed using western blot. There was a reduction in the expression of NQO1 with the treatment of E2 compared to the DMSO control. There was no significant effect on either SULT 1A1 or SULT 1E1 with E2 treatment tested by western blot. But the gene expression of (B) SULT 1A1, (C) SULT 1E1 and (D) UGT 1A1 was induced with the treatment of E2 tested by qPCR. Each point represents an average of three experiments done in duplicates ± SD and t test was done to calculate the p values.

2.3.3. Estrogen and estrogen metabolites cause malignant transformation

MCF-10A cells were treated with E2 (1 μM) and DMSO for 3 weeks and plated on agar for 30 days and colonies were counted at 14 days and 30 days. A significant increase in the anchorage independent colony formation in E2 treated sample was observed starting around 21 days (Figure 7). Therefore 3 weeks on agar was used as the optimum condition for the anchorage independent colony formation assay and these time points were used for all the experiments described in the present study. During the optimization of the colony formation assay, ras oncogene mutated MCF-10A cells which have
malignant phenotype were used as the positive control. There was a significant increase in the colonies in ras oncogene mutated MCF-10A cells compared to the DMSO control (Figure 7).

Figure 7: (A) There was a time dependent increase in anchorage independent colony formation with E2 in MCF-10A cells. (B) Anchorage independent colony formation in ras onco-gene mutated MCF-10A, DMSO control and E2 treated MCF-10A cells in soft agar. There was a significant increase in the formation of colonies in E2 treated samples compared to DMSO control. Also there was a significant increased in anchorage independent colony formation in ras oncogene mutated MCF-10A cells compared to normal MCF-10A cells treated with DMSO.
In order to test the effects of estrogen and estrogen metabolites on anchorage independent colony formation, MCF-10A cells were treated with different concentrations of E2 and 4-OHE2. There were an increased number of colonies formed in the samples treated with 5 μM E2 compared to E2 1 μM samples. Also there was an increase number of colonies formed with 1 μM 4-OHE2 compared to 1 μM E2 treated samples. Further there was a dose dependent increase in the colony formation with the treatment of 4-OHE2 (Figure 8).

![Figure 8: Anchorage independent colony formation with different concentrations of E2 and 4-OHE2.](image)

There was an increased number of colony formation with 4-OHE2 compared to the same concentration of E2 treated samples. Also there was a dose dependent increase in the formation of anchorage independent colonies with both compounds. MCF-10A cells were incubated with the indicative compounds for 3 weeks followed by plating on agar for 3 weeks. Anchorage independent colony formation was analyzed and average number of colonies formed per cells plated in each well × 100 was calculated and represented as colony formation efficacy. Each point represents an average of triplicates ± SD and p values were determined with ANOVA.
2.3.4. Effect of ethinyl estradiol on malignant transformation

Ethinyl estradiol (EE) is the main active constituent in most of the formulations of combined oral contraceptive pills. There has been some new studies done to show that EE has beneficial effects in advanced breast cancer patients (194). Therefore EE was used as a potential candidate to test the effect on $E_2$ induced malignant transformation. MCF-10A cells were treated with $E_2$ (1 μM) in the presence and absence of EE (1 μM) for 3 weeks. After 3 weeks cells were plated on soft agar for another 3 weeks. Anchorage independent colony formation in soft agar was analysed after staining the colonies with crystal violet as previously described in materials and methods. There was a significant inhibition in the $E_2$ induced colony formation with EE (Figure 9).

![Figure 9: There was a significant inhibition in the $E_2$ induced malignant transformation with EE (1 μM). MCF-10A cells were incubated with the indicative compounds for 3 weeks followed by plating on agar for 3 weeks. Anchorage independent colony formation was analyzed and average number of colonies formed per cells plated in each well × 100 was calculated and represented as colony formation efficacy. Each point represents an average of three independent experiments done in triplicates ± SD and the p values were calculated using ANOVA.](image-url)
2.3.5. Effect of E₂ on ROS formation in MCF-10A cells

MCF-10A cells were incubated with E₂ for 6 days and the ROS was labeled with CM-H₂-DCF-DA. There was an increased amount of ROS formation in the E₂ treated samples compared to the DMSO control. It has been reported that estrogen induced ROS formation is concentrated to the nucleus in ER + cells (195). But in MCF-10A cells there was an even distribution of ROS in the cytoplasm and nucleus without any concentration into the nucleus (Figure 10). It could be possible that since MCF-10A cells are considered ER(-) ER mediated accumulation of ROS into the nucleus was not observed in these experiments.

Figure 10: There was an increased formation of ROS in MCF-10A cells upon exposure to E₂ (1 μM) for 6 days. ROS formation was confined to the nucleus and an even distribution of ROS over the cytoplasm and nucleus was observed in MCF-10A cells. Cells were treated with the compounds for 6 days and ROS was labeled with labeled with CM-H₂-DCFDA (10 μM) for 30 min. Nuclei were labeled with Hochest nuclear dye.
**2.3.6. Effect of CYP1B1 gene knock down on estrone methyl ether formation.**

**Figure 11:** CYP 1B1 knock down significantly inhibited the formation of 4-MeOE₁ in MCF-10A cells. CYP 1B1 gene was knock down with siRNA and cells were treated with E₂ for 3 days. Cell media was collected and analyzed for 4-MeOE₁ formation with LC/MS-MS. Each point represents an average of two different experiments done in duplicates ± SD and p value was calculated using ANOVA.

*CYP1B1* gene was knocked down with siRNA and cells were treated with E₂ (1 μM) for 3 days before cell media was tested for 4-MeOE₁ using LC/MS-MS. Wild type MCF-10A cells was treated with E₂ for 3 days and used as the positive control in this experiment. Scrambled sequence of siRNA was used as the negative control in all the experiments. After siRNA mediated knock down was done, western blotting was done to confirm there was no CYP450 1B1 protein present in the cells. There was a significant inhibition in the 4-MeOE₁ formation in the *CYP1B1* knock down samples compared to the wild type MCF-10A cells (Figure 11). But the inhibition of 4-MeOE₁ formation was around 50%, suggesting that may be there are still CYP1B1 active in the cells without knocking down by siRNA or the
complete knock down was not possible with the present siRNA protocol specific for *CYP1B1*. Also it could be possible that there are other CYP450 enzymes responsible for 4-hydroxylation of E₂ in this cell line other than CYP450 1B1.

**2.3.7. Translocation of AHR into the nucleus by E₂ and 4-OHE₂ in MCF-10A cells**

Nuclear translocation of AHR with E₂ and 4-OHE₂ was analyzed in MCF-10A cells after treating the cells for 24 h with the compounds. 3-MC was used as the positive control while DMSO was used as the vehicle control. Some of AHR was translocated into the nucleus with 3-MC while DMSO did not have any effect. Both E₂ and 4-OHE₂ increased the AHR translocation into nucleus in MCF-10A cells (*Figure 12*). It could be possible that the treatment time was too long and not optimized for 3-MC and after 24 h most of the AHR translocated back to the cytoplasm.
Figure 12: Nuclear translocation of AHR with E$_2$ and 4-OHE$_2$ treatment in MCF-10A cells. E$_2$ and 4-OHE$_2$ could translocate AHR into the nucleus after 24 h. DMSO was used as the negative control in the experiment. MCF-10A cells were treated with 3-MC (1 μM) E$_2$ (1 μM), 4-OHE$_2$ (1 μM) and DMSO (0.01%) for 24 h. Cells were fixed and anti-AHR antibodies were used to label AHR in the cells. Nuclei were labeled with DAPI and the cells were imaged using Zeiss 510 META laser scanning confocal microscope.
2.4. Discussion and conclusions

Breast cancer has been a leading cause of death among women all over the world. Long term exposure to estrogens will increase the risk of developing hormone dependent cancer. There is a timely necessity to find new therapeutic measures in the treatment and prevention of breast cancer. In order to find new therapeutic approaches it is essential to understand the mechanism of estrogen carcinogenesis. Estrogen dependent cancer formation can occur either by increased proliferation of estrogen sensitive cells mediated via activation of ER (hormonal pathway); and/or increased formation of reactive electrophilic estrogen metabolites (chemical pathway) which can cause genotoxicity and ultimate carcinogenesis. The present study mainly focused on understanding the mechanism of estrogen chemical carcinogenesis in breast epithelial cells and discovering new biomarkers to identify the carcinogenicity.

Estrogen chemical carcinogenesis is mediated via oxidative estrogen metabolism leading to formation of highly reactive, electrophilic estrogen quinones (95). In breast epithelial cells estrogens can be metabolized to 4- and 2-hydroxy catechols in the presence of CYP450 1B1 and 1A1, respectively. Both of these catechols can further oxidized to form highly reactive semiquinones and quinones. The catechols can go through phase II detoxification mechanisms and conjugate with sulfate groups and glucuronic acid groups to increase water solubility and reduce toxicity. Conjugation of methyl groups to catechol estrogens will reduce the reactivity and toxicity by preventing quinone formation via oxidation. Since the stability of catechol estrogens in cell media was low, in the present study; formation of 4 and 2 methyl ether metabolites were measured in cell media assuming that their production was reflective of catechol estrogen formation (196). Liquid chromatography tandem mass spectrophotometry (LC/MS-MS) was used to analyze the metabolites in cell media, since it is a specific method compared to other methods used for analysis of estrogen metabolites such as radioimmunoassay (RIA) and ELISA (197). Catechol estrogen formation and DNA adduct formation in MCF-10F cells has been measured previously with pre-exposure to TCDD (2,3,7,8- tetrachlorodibenzo-p-dioxin) to induce CYP450s 1B1/1A1 and Ro-41-0960 to inhibit COMT (126). But less work has been done so far to understand the mechanism of estrogen
metabolism in MCF-10A cells without any enzyme induction or inhibition. In the present study, extraction of estrogen metabolites with dichloromethane made it more efficient compared to previously use solid phase extraction, while derivatization with dansyl chloride enhanced the ionization ability in the triple quadruple mass spectrometer leading to higher sensitivity. Measurement of estrogen metabolites can be used as an important biomarker in breast cancer since the quantification can be done easily and the inhibition of methyl ether formation correlates with the inhibition of carcinogenesis in breast epithelial cells.

In order to understand the mechanism of estrogen chemical carcinogenesis, an ER(-), immortalized, non tumorigenic human breast epithelial cells (MCF-10A) were used as the model system (166), since the interference from hormonal pathway is minimal. Estrogen metabolism and DNA adduct formation has been previously studied in MCF-10F cell model (126), which is another member of MCF-10 family.

Estrogen metabolizing enzymes play a major role in estrogen chemical carcinogenesis. Phase I metabolizing enzymes, CYP450 1B1 and 1A1 oxidize estrogens to their catechols which can get further oxidized to reactive quinones. Most of the carcinogens and toxins tend to induce these phase I metabolizing enzymes in order to activate the respective compounds. The potent carcinogen, TCDD (2,3,7,8-tetrachlorodibenzodioxin) induces CYP450 1B1 and increase the formation of 4-hydroxycatechol in estrogen treated MCF-10F cells (126). In the present study when CYP1B1 was knock down with siRNA, there was a significant inhibition in estrogen catechol formation (Figure 11), showing that CYP450 1B1 is the major phase I enzyme involved in E2 metabolism in MCF-10A cells. And also it was observed that E2 induced both CYP450 1A1 and 1B1 in a time dependent manner in MCF-10A cells (Figure 5). It has been reported that CYP1A1 gene is an inducible gene where as CYP1B1 is a constitutive gene (198). In ER positive cells, induction of CYP450 1B1 is ER mediated since there is an estrogen response element (ERE) in the CYP1B1 promoter region (128). Also it has been reported that polymorphisms in CYP1B1 gene in endometrial cancer cells positively correlated to the expression of ERα and ERβ tested in cancer patients (199). These previous reports demonstrate that it could be possible
to have a cross talk between ER and CYP1B1 gene in ER (+) cells. However, since the influence of ERα is minimal in MCF-10A cells, the mechanism of induction of CYP450 1B1 could be mediated through ERβ, AHR, or other orphan receptor in the present study. Beedanagari et. al showed that the induction of CYP1A1 and 1B1 genes by dioxins can be mediated via the formation of aryl hydrocarbon receptor (AHR) and RNA polymerase II complex (200). Yet a study done by Iwanari and colleagues showed that the induction of CYP1 family enzymes by PHA and NPHAs is independent to the expression level of AHR, ARNT, ERα or ERβ in different cell lines (201). Further experiments should be done to find out the molecular mechanism of induction of CYP enzymes in MCF-10A cells by E2.

Phase II/conjugative metabolizing enzymes play an important role in detoxification of estrogens and estrogen metabolites. It has been reported that COMT, SULT, and UGT are the main phase II detoxification enzymes involved in estrogen metabolism. In the present study, the expression of SULT1 family was tested; since it was previously reported that SULT1 izozymes are the prominent SULT enzymes in MCF-10A cells. There was an induction in the expression of SULT1 proteins with E2, yet the effect on SULT1A1 and SULT1E1 expression was minimal as tested by western blotting (Figure 6). Messenger RNA (mRNA) levels of SULT1A1 and SULT1E1 were analyzed to understand the effect of E2 on gene transcription and there was an induction in both SULT1A1 and SULT 1E1 mRNA levels with the treatment of E2 (Figure 6). SULT 1A1, 1A2, 1A3, 1C4, and 1E1 can use catechol estrogens and methoxyestrogens as substrates, while SULT 1E1 has the strongest sulfation ability (141). Further, SULT1E1 is widely studied due to its importance in steroid metabolism (105). Although UGT 1A1 is involved in the phase II metabolism of estrogen and estrogen metabolites, in the present study protein expression of UGT1A1 was below the detectable levels of UGT1A1 antibody used in western blot experiments. Messenger RNA (mRNA) levels of UGT1A1 were also low, yet detectable and there was a significant induction in mRNA levels with E2 treatment compared to the control (Figure 6). It was previously reported that the induction of SULT and UGT enzymes with octachlorostyrene was mediated via the orphan receptor, constitutive androstaner receptor (CAR) in liver cells (131). CAR expression is high in the digestive track and surrounding tissues compared to breast tissues (202). Also in the present
study the expression of CAR was below the detection level of western blotting suggesting that the possibility of SULT and UGT gene induction via CAR is minimal. There was an inhibition in the expression of NQO1 with E\textsubscript{2} treatment (*Figure 6*) suggesting that the conversion of quinone to catechol is attenuated in the presence of E\textsubscript{2}. NQO1 has been used as a key enzyme in the studies of chemoprevention, therefore the inhibition in NQO1 expression by E\textsubscript{2} could be an indication of increased quinone production and carcinogenicity by E\textsubscript{2} in this cell line.

Malignant transformation of normal cells to a cancer phenotype is assessed by the ability to form anchorage independent colonies in soft agar. MCF-10A cells can get transformed into a malignant phenotype upon exposure to estrogen and estrogen metabolites (167, 203, 204). Previous studies have shown that pre treatment for 4 weeks prior to maintaining the cells on agar for 4 weeks is necessary before counting the colonies (192). In the present study that method was optimized to get significant results by pre-treating the cells for 3 weeks prior to plating them on agar for 3 weeks. During the method development, ras onco-gene mutated MCF-10A cells were used as the positive control in anchorage independent colony formation assays since it showed a malignant phenotype without any treatment. There was a significant increase in the number of colonies in ras onco-gene mutated cells compared to the DMSO control. Also there was a significant increase in the anchorage independent colony formation with E\textsubscript{2} treatment (*Figure 7*). Present study shows that MCF-10A cells can get transformed into a malignant phenotype in the presence of E\textsubscript{2} and 4-OHE\textsubscript{2} in dose dependent manner (*Figure 8*). Also it has been shown that there is a dose dependent and time dependent effect on malignant transformation with E\textsubscript{2} in MCF-10A cells. Anchorage independent colony formation can be used as an important biomarker in the estrogen carcinogenesis in MCF-10A cells since it can be predictive of the tumorigenicity.

Oxidation of estrogens to catechols and quinones and the reduction of quinones to catechols will create a redox cycle which can lead to the generation of reactive oxygen species (ROS). Increased formation of ROS is indicative of oxidative stress and associated with carcinogenesis (189, 205). In the present study, it was observed that there is an increased production of ROS in the E\textsubscript{2} treated cells compared to DMSO treated cells (*Figure 10*). In ER (+) cells, estrogen induced ROS formation is mainly
confined in the nucleus since ER act as a “Trojan horse” to carry estrogens/estrogen metabolites into the nucleus (195). But in the present study, since MCF-10A cells are ER(-), ROS accumulation was not limited to the nucleus (Figure 10). ROS generation by E2 is time dependent and it was previously reported that there is no ROS production was observed when S30 and MDA-MB-231 cells were treated with E2 for 5 min (195). Since estrogens has to metabolized to catechols and then into quinones to initiate redox cycling, generation of ROS can be delayed with E2 treatment compared to the cells treated with the catechol. In the present study MCF-10A cells were treated for 6 days before analyzing for ROS. This is the first report to show that there is generation of ROS in MCF-10A cells upon exposure to E2 (1 μM) for 6 days. Estrogen induced generation of ROS could also be used a possible biomarker to predict the estrogen carcinogenicity.

In conclusion, E2 could induce malignant transformation, ROS formation in MCF-10A cells and could also modulate phase I and phase II metabolizing enzymes to modulate oxidative estrogen metabolism. Additionally this is the first study to show that there is a time dependent induction in the CYP450 1B1 and 1A1 expression with the treatment of E2 in MCF-10A cells. This study demonstrates that estrogen chemical carcinogenesis is mediated via oxidative estrogen metabolism and can be analyzed by measuring estrone methyl ether production, ROS generation, and anchorage independent colony formation. Therefore these factors could serve as useful biomarkers in estrogen carcinogenesis and modulation/attenuation of estrogen carcinogenesis could be achieved via manipulating them. Further studies should be done to understand the receptors and molecular targets of estrogen in human ER(-) breast epithelial cells.
Figure 13: Possible mechanism of estrogen inducing genotoxicity in breast epithelial cells. $E_2$ induces the expression of CYP450 1B1 and 1A1 in a time dependent manner. $E_2$ induces the reactive oxygen species generation in MCF-10A cells.
3: PREVENTION OF ESTROGEN CARCINOGENESIS BY BOTANICAL DIETARY SUPPLEMENTS

3.1. Introduction

3.1.1. Dietary supplements and women’s health

Botanical dietary supplements (BDS) are not regulated by Food and Drug Administration (FDA) and they are not intended for diagnosis, treatment, or prevention of a disease (206). Therefore substantial inconsistency in the content, dosage, and purity of these BDS can occur while leaving the question of safety and efficacy totally unanswered. Nonetheless, many surveys done in US and other western countries show that there is a recent trend, especially among western women, to turn to alternative medicine (207-209). Hormone replacement therapy (HRT) for post menopausal symptom relief had unexpected outcomes and side effects, leaving women in search for an alternative to HRT with reduced undesirable side effects. As a result, the recent trend in using alternative medicine among women have directed them to over the counter BDS for post menopausal symptom relief. It has been shown in a randomized, double blind clinical trial that red clover and black cohosh, which has been used in the relief of hot flashes for years by women, had no significant effect compared to the placebo group (210). In order to find a better alternative for HRT, research has been done to discover phytoestrogens with minimal undesirable side effects. However, due to lack of knowledge and less research done regarding these widely used botanicals and phytoestrogens, it makes a lot more difficult to decide which botanicals to be used as an alternative to HRT. Among the phytoestrogens discovered so far, genistein from soybean and in red clover as well as 8-prenyltaurine (8-PN) from hops are important because these botanicals could be useful for menopausal symptom relief. Methanol extracts from hops (Humulus lupulus L.) and red clover (Trifolium pretense L.) showed competitive binding to ERα and ERβ (211). Also these extracts showed estrogenic activity when tested in cultured endometrial cells as indicated by the induction of alkaline phosphatase activity (211). There is evidence to show that soy isoflavones, especially genistein could alleviate hot flashes associated with menopause and could be used as an alternative to HRT (212).
However, there is a growing body of evidence which showed that phytoestrogens available as soy food, soy extracts, and red clover extract do not improve hot flashes or other menopause related symptoms in women (213, 214). A recently done systemic review and meta analysis of randomized controlled trials about the use of soy flavonoids provided contradictory results. In one review, it was reported that extracted or synthesized soybean isoflavones are significantly more effective than placebo at reducing the frequency and severity of hot flashes (215). Another review published in the same journal showed that there is no significant effect on alleviation of post menopausal hot flashes with soy isoflavones compared to the placebo control, but long term use of a diet rich in soy isoflavones may reduce the risk of ovarian and endometrial cancer (216). Also there are reports showing that genistein and a synthesized genistein metabolite, 6-carboxymethyl genistein could be estrogen agonists, partial agonists, and antagonists in a tissue specific manner, suggesting that they may possess SERM like activity (217). Phytoestrogens could be a better alternative for HRT in post menopausal symptom relief yet further studies should be done to understand the safety, efficacy, proper dosage and duration of the treatment.

3.1.2. Botanicals in the prevention and treatment of cancer

Botanicals have been used in the prevention and treatment of diseases throughout history. There are numerous reports showing that there is a positive correlation between consumption of vegetables and fruits and cancer prevention (218, 219). Vegetables had superior effect compared to fruits in the prevention of gastrointestinal cancer, especially colorectal cancer (220). Many case control and cohort studies showed that people who eat about five servings of vegetables and fruits a day have approximately half the risk of developing cancer compared to those who eat fewer than two servings per day. NCI have identified several plant based foods with cancer prevention abilities, including ginger, garlic, onion, turmeric, tomatoes, and cruciferous vegetables. In vitro and in vivo research is underway to evaluate the ability of cancer prevention with vegetables and fruits, since it will be easier to prevent cancer with an edible plant material than with a pill (221). Research has extended to isolate the possible compounds that possess chemopreventive properties in vegetables and fruits. Thus discovery of compounds such as lycopene from tomatoes, resveratrol from grapes, curcumin from turmeric, [6]-gingerol from ginger,
diallyl sulphide from garlic, genistein from soybean, and sulforaphane from broccoli pioneered the plant based cancer prevention studies (221-223). Among the plant based compounds under investigation for cancer prevention ability, resveratrol plays a major role since there are several reports showing that resveratrol can inhibit cancer formation in cell culture models (125, 223-225). It has been reported that resveratrol can inhibit estrogen induced malignant transformation, DNA adduct formation, and oxidative estrogen metabolism in breast epithelial cells (125, 224, 225). The cancer preventive ability of resveratrol is achieved by the inhibition of hydroxylation of estrogens to catechols mediated via down regulating the expression of CYP450 1B1 and 1A1 in human breast epithelial cells (127). Another study showed that resveratrol can down regulate DNA repair genes to arrest the cell cycle in human breast cancer (MCF-7) cell line, sensitizing the cells to anticancer drugs and making it a possible candidate to use in combination therapy (226). However, the use of botanicals and chemotherapy at the same time should be done with caution, since the active ingredients in botanicals could be counterproductive to prescription chemotherapy drugs (227). There are reports showing that resveratrol can scavenge ROS to reduce oxidative stress and DNA damage and it also has significant inhibitory effect on NF-κB pathway, suggesting that antioxidant effect of resveratrol could contribute to cancer prevention (228). Prevention of cancer can also be achieved via induction of Nrf2 pathway with botanicals and compounds isolated from botanicals. Since the Nrf2/keap1 pathway controls phase II detoxification enzyme expression; induction of the Nrf2 pathway could increase the production of detoxification enzymes leading to increased formation of phase II conjugates and ultimate chemoprevention (164, 165). Sulforaphane found in broccoli is one of the compounds that can be used to induce Nrf2 pathway (229). Since soy isoflavone genistein showed bladder cancer cell growth inhibition in preclinical studies via inhibiting phosphorylation of epidermal growth factor receptor (EGFR), further studies were done to determine the effect on bladder cancer individuals. In a phase 2 clinical trial, it was observed that there is a reduction in the p-EGFR with a lower dose (300 mg/day) of genistein but not with a higher (600 mg /day) dose (230). Studies done in murine models showed that there is chemopreventive activity of genistein on prostate and mammary cancer (231). There should be more research done before initiating clinical trials with
botanicals, in order to understand pharmacokinetics, drug interactions, dosage, safety, and efficacy (232, 233).

Black cohosh \([Cimicifuga racemosa (L.) Nutt.; syn Actaea racemosa (Nutt.) L]\) and hops \((Humulus lupulus L.)\) are currently popular remedies for postmenopausal women as natural alternatives to HRT. Their mechanisms of action are not completely known; however, estrogenic activity for hops and serotonergic effects for black cohosh have been reported. Hops contain numerous active compounds including xanthohumol (XH) that has been shown to have chemopreventive activity (234). Present study focused on understanding the potential of widely used botanical, hops \((Humulus lupulus L.)\) to be used as a breast cancer chemopreventive agent.
3.2. Materials and methods

3.2.1. Plant materials and phenolic compounds

Authentic *C. racemosa* (L.) Nutt. (syn. *Actaea racemosa* L., black cohosh) rhizomes/roots (BC #192) were acquired through Naturex (South Hackensack, NJ) and were botanically verified and characterized by the UIC/NIH Center for Botanical Dietary Supplements (210). Hops (*Humulus lupulus*) extract used for the experiments was an ethanol extract of spent hops dispersed in kieselguhr (plant materials were extracted with ethanol after supercritical CO$_2$ extraction of pelletized strobiles of *Humulus lupulus* cv. Nugget), which was obtained from Hopsteiner (Mainburg [Germany]/New York). The kieselguhr was removed by methanol filtration. Quantitative LC-MS analysis using authentic reference compounds as calibrants revealed that this hops extract contained 5.4% XH (xanthohumol) and 0.084% 8-PN (8-prenylnaringenin) (235). XH was isolated and purified (>99.5% purity both by qHNMR and LC-MS) as described previously (234). 8-PN was synthesized and purified (95.0% purity by qHNMR) using the modified literature procedure as previously reported (235).

3.2.2. Analysis of estrogen metabolites with LC/MS-MS

MCF-10A cells were incubated with E$_2$ (1 µM) in the presence or absence of hops (5 µg/mL) and black cohosh (20 µg/mL) for 6 days. Since, 20 µg/mL of hops showed toxicity in MCF-10A cells, lower concentrations of hops (5 µg/mL) was used for all the experiments. Dose dependent inhibition of estrogen metabolism by hops was performed by incubating MCF-10A cells with different concentrations of hops (1-10 µg/mL) in the presence of E$_2$ (1 µM). The effect of the compounds isolated from hops; XH and 8-PN on estrogen metabolism was also studied. The dose dependent effect of XH (0.1 - 5 µM) and 8-PN (0.5 - 5 µM) was tested in the presence of E$_2$ (1 µM). Estrogen metabolites were quantified using Analyst software (Applied Biosystems, Forster City, CA). Peak areas of 2 and 4-MeOE$_1$ were normalized against 2- MeOE$_1$-d$_4$ internal standard to obtain the relative peak areas. 2 and 4-MeOE$_1$ relative peak areas in E$_2$ treated sample were considered as 100% and all the other samples were normalized against that and represented as percentage MeOE$_1$ formation.
3.2.3. Immunoblotting

Protein expression of CYP450 1B1 and 1A1 was analyzed using western blot experiments as previously described (190). Briefly, protein lysates were prepared and electrophoresis was done to separate proteins. Each lane was loaded with 30 µg of total protein as determined by BCA assay. Anti-CYP450 1B1, anti-CYP450 1A1, anti-COMT, anti NQO1 and anti-β-actin antibodies were used as primary antibodies. Blots were incubated with primary antibody overnight at 4 °C and with secondary antibody for 1 h at room temperature. Blots were visualized using chemiluminescence substrate (Thermo scientific, Rockford, IL). Imaging and analysis was done using FluroChem software (Cell Biosciences, Santa Clara, CA). β-Actin level was measured as a gel loading and transferring control. Each protein band density was normalized to the respective β-actin band density and was represented as the relative protein expression. Three independent experiments were done to get the average and the results were represented as average ± SD.

3.2.4. Enzyme activity assays for CYP450 1B1 with hops

Human recombinant CYP450 1B1 isozymes with CYP450 reductase were purchased from Sigma. CYP450 1B1 enzyme activity was first evaluated using E₂ as the substrate. Reaction mixture (1 mL) containing, recombinant CYP450 1B1 (10 pmol), E₂ (5 µM), potassium phosphate buffer (50 mM, pH = 7.4) and either hops (20 µg/mL) or 8-PN (1 µM) was pre-incubated at 37 °C for 5 min. The reaction was initiated by adding NADPH (1 mM) into each reaction mixture and incubated for 1 h at 37 °C. The reaction was quenched by adding 100 µL of acetonitrile at 0 °C and protein was removed by centrifugation (10,000 rpm for 10 min). Ascorbic acid (2 mM) was added in to each sample before 4-OHE₁-d₄ (5 nM) was added as the internal standard. Estrogen metabolites were extracted with dichloromethane (2 × 2 mL) and derivatized with dansyl chloride and analyzed by LC/MS-MS as described above for the metabolism experiments. 4-OHE₂ peak area was normalized to the peak area of internal standard in each sample to cancel out the extraction differences in each sample. 4-OHE₂
formation with E₂ was taken as 100% and other samples were normalized to E₂ sample and represented % 4-OHE₂ formation.

Inhibition of CYP450 1B1 activity was further confirmed using the ethoxyresorufin O-dealkylase assay (EROD) as described previously (35) with minor modifications. Briefly, recombinant CYP450 1B1 was incubated with E₂ (1 µM) and NADPH (1 mM) in potassium phosphate buffer (50 mM, pH = 7.4) in the presence and absence of different concentrations of hops (1-40 µg/mL). Activity of CYP450 1B1 was measured after incubating the reaction mixture at 37 °C for 10 min and determined as percentage CYP450 1B1 activity calculated from the resorufin standard curve.

3.2.5. Anchorage independent colony formation assay

MCF-10A cells were treated with E₂ (1 µM) in the presence and absence of hops (5 µg/mL), 8-PN (50 nM) or XH (1 µM) for 3 weeks. Cells were treated with the compounds twice a week. DMSO (0.01%) was used as the vehicle control in the experiments while E₂ (1 µM) was used as the positive control. Anchorage independent colony formation assay was performed as mentioned in chapter 2 material and method section.
3.3. Results

3.3.1. Effect of hops and black cohosh on estrogen metabolism.

MCF-10A cells were treated with E$_2$ (1 µM) in the presence or absence of either hops (5 µg/mL) or black cohosh extracts (20 µg/mL) for 6 days and cell media was analyzed for estrone methyl ether. Lower concentrations of hops (5 µg/mL) were used in the metabolism experiments as cell viability experiments indicated that 20 µg/mL of hops was toxic in MCF-10A cells (LC$_{50}$ = 11 ± 0.5 µg/mL). Hops (5 µg/mL) significantly reduced (p < 0.005) the formation of both 2- and 4-estrone methyl ethers (Figure 14). But black cohosh did not show any significant effect on estrogen metabolism in MCF-10A cells (Figure 14).

![Figure 14: Representative positive ion-electrospray selective reaction monitoring (SRM) chromatograms of 2-MeOE$_1$ and 4-MeOE$_1$ in (A) hops (5 µg/mL) and (B) black cohosh (20 µg/mL) treated MCF-10A cell media samples. (A) hops significantly inhibited the formation of 2-MeOE$_1$ and 4-MeOE$_1$ while (B) Black cohosh had no significant effect on 2-MeOE$_1$ and 4-MeOE$_1$ formation. MCF-10A cells were treated with E$_2$ in the presence and absence of hops (5 µg/mL) or black cohosh (20 µg/mL) for 6 days. Cell media was collected and were analyzed by LC/MS-MS. Overlaid SRM chromatograms represent the formation of 2-MeOE$_1$ and 4-MeOE$_1$ in E$_2$ treated (line) and E$_2$ and (A) hops (dashed line), or (B) black cohosh (dashed line) treated samples.](image-url)
Since hops showed a significant inhibition in estrone methyl ether formation at 5 µg/mL further studies were done to understand the dose dependent effect of hops on estrone methyl ether formation. There was a dose dependent reduction in the formation of both 2 and 4-MeOE$_1$ with hops extract in MCF-10A cells (Figure 15).

![Graph showing dose dependent effect of hops on estrone methyl ether formation](image)

**Figure 15:** There is a dose dependent effect of hops on the formation of 2-MeOE$_1$ and 4-MeOE$_1$ in MCF-10A cells. Cells were treated with different concentrations of hops in the presence of E$_2$ (1 µM) for 6 days and cell media was collected and analyzed for the estrogen metabolites using LC/MS-MS. Each value represents the average of three experiments performed independently in duplicate ± SD.

### 3.3.2. Effect of hops on the expression of metabolizing enzymes.

Phase I metabolism of E$_1$ and E$_2$ is mainly mediated via CYP 1 family in breast epithelial cells (95, 236). It was previously observed that E$_2$ could induce CYP450 1B1 and 1A1 in a time dependent manner (Figure 5) in MCF-10A cells. To determine the effect of hops on E$_2$ induced P450 enzymes, MCF-10A cells were treated with E$_2$ (1 µM) in the presence or absence of hops (5 µg/mL) for 1, 3, and 6
days and protein expression was analyzed by immunoblotting. There was a significant time dependent
induction in CYP450 1B1 and CYP450 1A1 with E\textsubscript{2} treatment with maximum induction at day 6. Co-
treatment with hops (5 µg/mL) significantly inhibited the induction of both CYP450 1B1 and CYP450
1A1 (Figure 16). There was an induction in the expression of NQO1 in the presence of hops and the
induction was time dependent (Figure 17). There was no significant effect on COMT by either E\textsubscript{2} or hops
(Figure 17).

Figure 16: Hops inhibits E\textsubscript{2} induced (A) CYP450 1B1 and (B) 1A1 expression in MCF-10A cells
evaluated by western blotting. Cells were treated with E\textsubscript{2} in the presence and absence of hops for 6 days.
Proteins were extracted and tested for CYP450 1B1 and 1A1 at day 1, 3 and 6 using western blotting.
Each data point represents an average of three independent experiments done in duplicate ± SD and p
values were calculated using ANOVA.
Figure 17: (A) There was no significant effect on COMT with either E₂ or hops treatments. (B) There was an induction in the expression of NQO1 with hops co-treatment. Cells were treated with E₂ in the presence and absence of hops for 6 days. Proteins were extracted and tested for COMT and NQO1 at day 1, 3 and 6 using western blotting. Each data point represents an average of three independent experiments done in duplicate ± SD and the p values were calculated using ANOVA. Significant difference was represented when p < 0.05, *.

3.3.3. Effect of hops and its compounds on CYP450 enzyme inhibition.

Direct inhibition of CYP450 1B1 metabolism by hops extracts was analyzed and the data showed that toxic concentrations were necessary before any significant inhibition of estradiol metabolism or ethoxyresorufin O-dealkylation activity was observed. When human recombinant CYP450 1B1 activity was evaluated measuring the conversion of E₂ to 4-OHE₂ using LC/MS-MS, there was a significant inhibition observed in both hops (20 μg/mL) and 8-PN (1 μM) treated samples (Figure 18). But the hops
concentration necessary to show a significant inhibition was beyond the LC₅₀ of hops in breast epithelial cells (Figure 19). Also the 8-PN concentration that gave significant enzyme activity inhibition was beyond the dosage found in the hops extract which is usually in nano molar range.

Figure 18: (A) CYP450 1B1 activity is significantly inhibited by hops and 8-PN at higher concentrations. Recombinant human CYP450 1B1 isozyme was incubated with E₂ (5 µM), E₂ + hops (20 µg/mL) and E₂ + 8-PN (1 µM) and the formation of 4-OHE₂ was measured using LC/MS-MS. 4-OHE₂ formation with E₂ was taken as 100% and other samples were normalized to E₂ sample. Each value represents an average of two experiments performed independently in duplicate ± SD and the p values were determined by ANOVA. (B) Inhibition of CYP450 1B1 activity measured by ethoxyresorufin O-dealkylation assay. CYP450 1B1 activity was significantly inhibited by hops at a concentration which is toxic in a cellular system. Each data point represents an average of three experiments ± SD.
Inhibition of CYP450 1B1 activity was further confirmed by EROD assay using ethoxyresorufin as the substrate. A significant inhibition was observed at higher concentrations of hops (Figure 18) which are beyond the LC_{50} of hops in breast epithelial cells (Figure 19).

### 3.3.4. Cytotoxicity of hops

Cytotoxicity of hops extract in MCF-10A cells were evaluated using MTT assay. Cell viability was assessed after treating the cells with different concentrations of hops for 6 days in parallel to the metabolism experiments. It was found that the LC_{50} = 11 ± 0.5 µg/mL (Figure 19). Therefore the concentration which was giving a significant inhibition in estrogen metabolism (Figure 15) and malignant transformation (Figure 21) was within the non toxic region. However, the concentrations needed to inhibit CYP450 1B1 activity (Figure 18) was beyond the LC_{50} level.

![Graph showing MCF-10A cell viability with hops](image)

*Figure 19: MCF-10A cell viability with hops. MTT assay was performed in parallel to the metabolism experiments to assess the cytotoxicity of hops in MCF-10A cells. Each value represents an average of three experiments performed independently in duplicate ± SD.*
3.3.5. Effect of XH and 8-PN on estrogen metabolism.

Since hops showed a significant inhibition in estrone methyl ether formation, the hops compounds, XH and 8-PN, were further analyzed for their ability to inhibit estrogen metabolism in MCF-10A cells. It has been previously reported that XH exhibits chemopreventive activity (234) and 8-PN shows estrogenic activity (235). Since pure standards of these compounds were available, they were tested for their effect on estrogen metabolism. No significant reduction in the formation of 2- and 4-MeOE\(_1\) was observed with the co-treatment of different concentrations of XH in MCF-10A cells (Figure 20). In contrast, there was a significant reduction (p < 0.005) in the formation of 2- and 4-MeOE\(_1\) in the presence of nanomolar amounts of 8-PN (Figure 20).

![Figure 20: Effect of XH and 8-PN on estrogen metabolism in MCF-10A cells. 2-MeOE\(_1\) (open circles) and 4-MeOE\(_1\) (closed circles) formation was plotted against different concentrations of XH and 8-PN. MCF-10A cells were treated with E\(_2\) (1 µM) in the presence and absence of different concentrations of either XH or 8-PN for 6 days. Cell media was analyzed for estrogen metabolites using LC/MS-MS. There was a significant inhibition of both 2-MeOE\(_1\) and 4-MeOE\(_1\) formation in the presence of nanomolar concentrations of 8-PN. Each value represents an average of three experiments performed independently in duplicate ± SD.](image-url)
3.3.6. Effect of hops and its compounds on malignant transformation of MCF-10A cells.

MCF-10A cells can be transformed into a malignant phenotype and can gain the ability to form anchorage independent colonies in soft agar (237). When MCF-10A cells were treated for 3 weeks and plated on soft agar for 3 weeks, there was a significant increase in anchorage independent colony formation in the E\(_2\) treated sample compared to the negative control (0.01% DMSO). Co-treatment with hops (5 µg/mL) significantly inhibited (p < 0.005) E\(_2\) induced colony formation in soft agar. There was also a significant reduction (p < 0.0001) in colony formation with the co-treatment of 8-PN (50 nM). Although XH (1 µM) did not inhibit estrone methyl ether formation in MCF-10A cells it could significantly inhibit E\(_2\) induced malignant transformation in MCF-10A cells (Figure 21).

Figure 21: Effect of hops, 8-PN and XH on E\(_2\) induced malignant transformation in MCF-10A cells.

MCF-10A cells were treated with E\(_2\) (1 µM) in the presence and absence of hops (5 µg/mL), 8-PN (50 nM) or XH (1 µM). Treatments were continued for 3 weeks and cells were passaged once a week. At the end of 3 weeks, cells were plated and maintained on soft agar for 3 weeks and formation of colonies were observed and counted using an inverted microscope. Hops (p < 0.005), 8-PN (p < 0.0001) and XH (p < 0.005) significantly inhibited E\(_2\) induced malignant transformation in MCF-10A cells. DMSO treated (0.01%) samples were used as a negative control. Each point represents an average of triplicate ± SD and the p values were calculated using ANOVA.
3.4. Discussion and conclusions

Since women are turning to OTC botanicals for relief of menopausal symptoms without knowing the safety and efficacy of these botanical dietary supplements, there is a timely necessity to find safe and effective alternatives to HRT. Among the botanicals used in the alleviation of menopausal symptoms, hops and black cohosh are important as they are included in many remedies used by women. The present study focused on evaluating the breast cancer chemopreventive ability of these botanicals. We hypothesized that both hops and black cohosh could inhibit estrogen metabolism in such a way to attenuate malignant transformation in MCF-10A cells. However, this hypothesis was proven to be correct only for hops and not for black cohosh since hops could reduce oxidative estrogen metabolism and malignant transformation in MCF-10A cells.

Estrogen chemical carcinogenesis is mediated via oxidative estrogen metabolism. One potential mechanism of estrogen carcinogenesis is believed to be due to the formation of highly reactive, electrophilic estrogen quinones (95). Therefore the agents that can attenuate estrogen metabolism could have the potential to reduce estrogen carcinogenesis. The chemopreventive activities of hops and black cohosh have been investigated and ascribed to induction of detoxification enzymes (234) and antioxidant activity (238), respectively. In the present study hops but not black cohosh reduced the formation of estrone methyl ethers in MCF-10A cells (Figure 14). Methyl ether formation was assumed to be reflective of the amount of catechol formation in the cells and estrone methyl ethers were measured in cell media. Hops showed a dose dependent effect on the inhibition of methyl ether formation (Figure 15). It is considered that 4-hydroxycatechol is more mutagenic and genotoxic compared to 2-hydroxycatechol (97). Therefore inhibition of 4-hydroxylation over 2-hydroxylation is considered beneficial. But with hops, no selectivity was observed for the inhibition of 2 and 4 catechol formation; instead hops reduced both metabolites significantly starting at 5 μg/mL (Figure 15). There have been reports to show that botanicals such as resveratrol could reduce oxidative estrogen metabolism in MCF-10A and MCF-10F cells (127,
Similar modulation of estrogen metabolism has been reported for dietary berries in animal models (239).

There could be several possible mechanisms to explain the inhibitory effect of hops on estrogen metabolism. One of the mechanisms could be downregulation of CYP450 enzymes which convert E₂ to its catechols. It has been previously shown that E₂ induces the expression of CYP450 1B1 in MCF-7 cells through a mechanism involving both ERE and ERα (128). Furthermore, CYP450 1A1 expression could also be induced by E₂ in HEPA 1C1C7 cells via transcriptional regulation (240). Both of these CYP450 enzymes can be induced in MCF-10F cells by TCDD (126, 127). Compounds such as resveratrol could inhibit the up regulation of CYP450 1B1 and 1A1 in MCF-10A and MCF-10F cells to inhibit oxidative estrogen metabolism (125, 224, 241). In the present study, hops significantly inhibited E₂ induced upregulation of CYP450 1B1 and 1A1 in MCF-10A cells (Figure 16). The expression regulation of both CYP450 enzymes involved in estrogen metabolism depends on ER status in ER (+) breast epithelial cells (129). Since MCF-10A cells do not respond to estrogen via classical ERα genomic pathways and considered ER(-), the mechanism of CYP450 induction by E₂ could involve extra-nuclear ER, ERβ, or aryl hydrocarbon receptor (AhR) since these receptors are expressed in MCF-10 cells (242). Hops and the compounds isolated from hops showed chemopreventive activity in hepatic cells tested by QR assay (234) which is a widely used assay to test chemopreventive agents in vitro. When the cells were co-treated with E₂ and hops for 6 days there was an induction in the NQO1 expression relative to the E₂ alone treated samples (Figure 17). This is the first report to show that hops could induce the expression of NQO1 in human breast epithelial cells. Also there was no significant effect on the expression of COMT with any of the treatments in this study (Figure 17). Since there was no significant effect on COMT with any of the treatments, the assumption that the measurement of methyl ether formation is reflective of the amount of catechol formation is sensible.

Apart from the inhibition of upregulation of CYP450 enzymes by hops, it could be possible that estrogen metabolism is attenuated via activity inhibition of these enzymes. Resveratrol was reported to be an inhibitor of CYP450 1B1 and 1A1, with nanomolar potency (243). Enzyme kinetics experiments done
with CYP450 1 family enzyme supersomes showed that trans resveratrol could inhibit CYP450 1A1 and 1B1 with lower micromolar concentrations (244). However, similar studies in CYP450 1B1 and 1A1 supersomes, in which E2 was used as substrate, did not show inhibition below micromolar concentrations of resveratrol (127). Similarly, hops had no effect on CYP450 1B1 estrogen 4-hydroxylase or ethoxyresorufin O-dealkylase activity unless toxic concentrations were used (> 20 µg/mL, *Figure 18*). Moreover when CYP450 1B1 supersomes were incubated with E2 in the presence and absence of hops and 8-PN, significant inhibition in the formation of 4-OHE2 was observed only with very high toxic concentrations (*Figure 19*). Therefore it was confirmed that the mechanism of inhibition of estrogen metabolism by hops and 8-PN is not mediated via inhibition of CYP450 1B1.

Since hops showed a significant inhibition in oxidative estrogen metabolism in MCF-10A cells, isolated phenolic components from hops were further studied for their ability to attenuate estrogen metabolism in MCF-10A cells. XH is the most abundant and bioactive phytoconstituent of hops which has been previously studied extensively regarding its chemopreventive potential (234). But in the present study estrone methyl ether formation was not significantly affected with XH co-treatment in MCF-10A cells (*Figure 20*). In contrast, 8-PN which is a potent estrogenic compound isolated from hops (235), showed a significant inhibitory effect (*p* < 0.0005) even at nanomolar concentrations (*Figure 20*).

Transformation of a normal cell to a malignant phenotype will increase the ability to survive and form anchorage independent colonies in semi-solid media (237). MCF-10A and 10F cells could be transformed to malignant phenotype upon exposure to E2 and its metabolites (125, 192). It has been reported that E2 induced malignant transformation could be inhibited by botanical components such as resveratrol in human breast epithelial cells (125, 224). In the present study, there was a significant inhibition (*p* < 0.005) of E2 induced malignant transformation by hops (*Figure 21*). 8-PN which is a potent inhibitor of oxidative estrogen metabolism in MCF-10A cells also caused a significant reduction (*p* < 0.0001) in E2-induced malignant transformation when treated in nanomolar (50 nM) concentrations (*Figure 21*). Since 8-PN was used in low concentrations it is less likely that classical antioxidant effect plays a major role in the mechanism of inhibition of malignant transformation by 8-PN. It has been reported that 8-PN can act via
ERβ to modulate gene expression in rat brain cells (245). The mechanism of inhibition of oxidative estrogen metabolism and malignant transformation in MCF-10A cells by 8-PN could be through the inhibition of E2 upregulation of CYP450 1B1, which could be mediated via ER-β, extranuclear ER, or AhR. Surprisingly, XH which was not a significant inhibitor in methyl ether formation was also showed an inhibition in E2 induced malignant transformation in MCF-10A cells. Since there are evidence to show that there is an induction of QR activity with XH in liver cells (234), it could be possible that XH inhibits malignant transformation by inducing NQO1 activity and thereby reducing the formation of highly reactive quinone. It could also be possible that the antioxidant effects of XH are responsible for the inhibition of malignant transformation in MCF-10A cells since there are report to show that XH can act as an antioxidant in hepatic cells (246). However these interesting findings are to show that the complex nature of botanical extracts and to demonstrate that different compounds isolated from one extract can be responsible for different effects with diverse mechanisms in same cell type.

Hops extract and the compounds isolated from hops have been reported to have estrogenic activity in vitro and in vivo (247-249). In this study, we found that hops could significantly inhibit oxidative estrogen metabolism and E2 induced malignant transformation in breast epithelial cells. There has been reports showing that there could be similarities in the mechanism of action of SERMs and phytoestrogens (250). It could be possible that hops and the compounds in hops act as SERMs and has tissue or organ selectivity. Also it could be possible that the compounds in hops extract interact with different signal transduction pathways such as NF-κB to attenuate malignant transformation. It is reasonable to perform further investigations to find out the receptors these compounds act on and the signal transduction pathways they could interfere with. These findings may direct botanical research into a new direction and may lead to find out their effects in different perspectives.

In conclusion, hops extract inhibited estrogen oxidative metabolism and estrogen-induced malignant transformation in the MCF-10A model of mammary carcinogenesis. Hops significantly inhibit E2 induced up regulation of CYP450 1A1 and 1B1 in MCF-10A cells. A compound isolated from hops, 8-PN showed significant inhibition in estrone methyl ether formation while XH had no effect. Interestingly 8-PN and
XH both inhibited E2 induced malignant transformation suggesting that these two compounds may act via two different mechanisms. Further work should be performed to distinguish the site of 8-PN and XH acting to modulate estrogen induced malignant transformation, which could be a non-classical ER or AHR. However, these results suggest that it is worthwhile to further investigate hops in animal models and humans since it could be a potential breast cancer chemopreventive botanical dietary supplement.

Figure 22: Possible mechanism of action of hops acts as an inhibitor of estrogen carcinogenesis in human breast epithelial cells. E2 induces both CYP450 1B1 and 1A1 while there was significant inhibition of both enzymes by hops. Further, hops significantly induced the expression of NQO1.
4: PREVENTION OF ESTROGEN CARCINOGENESIS BY SERMS

4.1. Introduction

4.1.1. Mechanism of action of SERMs

Estrogen receptor (ER) mediated gene transcription and increased cellular proliferation in estrogen sensitive cells are the mechanisms of hormonal carcinogenesis (94). ERα can be found predominantly in breast, uterus, ovaries, liver and central nervous system; while ERβ is expressed in bone, brain, lung, endothelium, central nervous system and prostate (34, 251). ER can be activated upon binding of an ER ligand followed by conformational changes and triggering recruitment of co-activators to initiate gene transcription. Selective estrogen receptor modulators (SERMs) are estrogen receptor (ER) ligands that can mimic or oppose the effects of endogenous estrogen, in a tissue and organ specific manner. As indicated by the nomenclature, SERMs were designed to modulate estrogen activity by binding to ER in a tissue selective manner. Although SERMs can act as ER ligands, they lack the steroid structure seen in most of the typical estrogen ligands. SERMs are clinically significant class of compounds that can be broadly classified into five chemical groups; i.e. triphenylethlenes, benzo thiophenes, tetrahydronaphthlenes, indoles, and benzopyrans (60).

ER consists of three domains; i.e. AF-1, DNA binding domain (DBD), and ligand binding domain (LBD) while ER α ligands bind exclusively to C-terminal LBD of ER. Since ERα is abundant and regulate the development, differentiation and maintenance of reproductive, skeletal and cardiovascular tissues, compounds that can modulate ER α activity can be used to treat breast cancer, osteoporosis and cardiovascular diseases (252). Molecular mechanism of SERM action has been explained by Shiau et.al. using crystal structures of agonist and antagonist bound ER (253). According to that study, upon binding of an agonist a conformational change can occur in the helix 12 in LBD, facilitating recruitment and interaction of co activators leading to gene transcription. On the other hand, binding of an antagonist can trigger a change in the orientation of helix 12 to prevent binding and recruitment of co-activators whilst binding of co repressors are not hindered leading to transcriptional repression (253). However, unlike estrogens which are uniformly agonists and antiestrogens which are uniformly antagonists, SERMs have
agonist and antagonist effects in a tissue and organ selective manner. Three mechanisms can be used to explain the tissue selectivity of SERMs; differential expression and distribution of ER, conformational changes in the ER upon binding of a ligand, and differential expression and binding of co-regulators to ER (254). Considering the ability to be tissue specific, the concept of “an ideal SERM” put forward as a timely necessity as an alternative to hormone replacement therapy. Such ideal SERM should be estrogenic on bone to reduce postmenopausal osteoporosis, on the cardiovascular system to reduce stroke and other cardiovascular diseases, while having antiestrogenic effects on breast and endometrium.

4.1.2. SERMs in women’s health and cancer

Women are using hormone replacement therapy (HRT) seeking relief from post menopausal symptoms such as osteoporosis, hot flashes, anxiety, and mood changes. However, conventional HRT which is based on equine estrogens could have undesirable side effects causing women to look for alternative options (255). Since SERMs can be estrogenic in bone, heart, and CNS they could be a better choice to replace HRT. Tamoxifen is a first generation triphenylethylene SERM used clinically in the treatment of ER(+) breast cancer because it antagonizes estrogenic action in the breast, thereby preventing the proliferation of cancer cells in this tissue (256, 257). During clinical use it was observed that tamoxifen could reduce osteoclast activity and osteoporosis which was the opposite result investigators were expecting (258). Also there was a reduction in the vertebral fracture risk in postmenopausal women with osteoporosis when treated with tamoxifen (259). These observations gave the idea of tissue selectivity of SERMs and led to the development of more SERMs with modified structures. However, tamoxifen also behaves as a partial agonist in the endometrium, where it stimulates cell proliferation and promotes tumor formation (63, 260). This led to the development of new SERMs with less side effects (261). Also it was reported that most of the triphenylethylene SERMs including toremifene, droloxifene, idoxifene, and clomiphene act as partial agonists in the endometrium, which minimized the use of these SERMs (262). Nonetheless, historically tamoxifen remains the anti-hormonal therapy of choice for the treatment of ER(+) breast cancer, and still it is the treatment of choice for premenopausal ER(+) breast cancer treatment (263). It was reported that the endometrial cell toxicity of Tam is mediated via oxidative
bioactivation of Tam to a highly reactive classical quinone methide which is capable of damaging DNA by electrophilic reactions (264).

Raloxifene (Ral), a second generation benzo thiophene SERM (BT-SERM) developed for the treatment of postmenopausal osteoporosis, showed a reduction in breast cancer risk in post menopausal women (265). The study of tamoxifen and raloxifene (STAR) which was done to understand the effects of Tam and Ral on primary prevention of breast cancer in postmenopausal women, revealed that raloxifene is as effective as tamoxifen in reducing the risk of invasive breast cancer with a lower risk of endometrial cancer occurrence (66). However, long term use of raloxifene has been correlated with an increased risk of fatal stroke and venous thromboembolic events (266, 267). Another benzo thiophene SERM, arzoxifene was also antagonistic in both endometrium and mammary gland in animal models (69). Bazedoxifene, an indole SERM which is in clinical use in the treatment of osteoporosis in Europe, was also reported to have antagonistic effects on both breast and endometrium (262). It has been reported that Baze can inhibit hormone independent breast cancer cell growth and also could down regulate ER α and cyclin D1 (268).

In the present study, three clinical and pre clinical BT-SERMs, Ral, desmethy larzoxifene (DMA), and 4’-FDMA were tested for their effect on oxidative estrogen metabolism in breast epithelial cells. In addition, two clinical SERMs, Baze and the active metabolite of tamoxifen (4-hydroxytamoxifen) were also tested for their effect on oxidative estrogen metabolism in MCF-10A cells. It was hypothesized that clinical and pre clinical SERMs could attenuate estrogen carcinogenesis by inhibiting oxidative estrogen metabolism in human breast epithelial cells. However, this hypothesis was proven to be correct only for Ral, DMA, and Baze among the SERMs tested.
4.2. Materials and methods

4.2.1. Cell lines and cell culture condition

MCF-10A cells were used in all the experiments and the cell culture conditions were the same as previously described in chapter 2, materials and method section.

4.2.2. Chemicals and reagents

All chemicals and reagents were obtained from Sigma (St. Louis, MO) or Invitrogen (Carlsbad, CA) unless stated otherwise. Antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and cell signaling technology (Boston, MA) and Sigma (St. Louis, MO). All the standard compounds of estrogen metabolites were obtained from Steraloides Inc. (Newport, RI). Deuterated estrogens were obtained from CDN isotope (Pointe-Claire, Quebec) and used as internal standards in estrogen metabolism experiments. Commercially available SERMs were obtained from Sigma and all the other SERMs were synthesized in the lab as previously described (269, 270).

![Figure 23: Chemical structures of clinical and pre-clinical SERMs used in the study](image_url)
4.2.3. Analysis of estrogen metabolites using LC/MS-MS

MCF-10A cells were incubated with E₂ (1 µM) in the presence or absence of SERMs (1 µM) for 6 days. Treatments were renewed every 3 days. Since, DMA and Ral showed a significant inhibition of estrogen metabolism at 1 µM in MCF-10A cells, a dose response was performed for those two SERMs. Cells were treated with different concentrations of DMA and Ral (0.1 - 2.5 µM) in the presence of E₂ (1 µM). Sample preparation and analysis was done using the described by Xu et al. (191) with minor modifications as previously described in the chapter 2 materials and methods.

Enzymatic hydrolysis of the cell media was done as previously described (191) with minor modifications. Briefly, MCF-10A cells were plated in 6 well plates with 3 mL of media in each well. Cells were treated with E₂ (1 µM) in the presence or absence of SERMs (1 µM) for 6 days and treatments were renewed every 3 days. Cell media was collected every 3 days pooled together to get total of 6 mL for each sample. Internal standard (2-MeOE₁-d₄) was added into each sample before further processing. Enzyme hydrolysis buffer was prepared as previously described (191) which contained L-ascorbic acid, β-glucuronidase and sulfatase in 0.15 M sodium acetate buffer (pH = 4.6). Equal amounts (6 mL) of hydrolysis buffer was added into each cell media sample (6 mL) and incubated overnight (16 h) at 37 °C. Samples were extracted into dichloromethane and analyzed using LC/MS-MS as previously described (190).

4.2.4. Immunoblotting

MCF-10A cells were treated with E₂ (1 µM) in the presence and absence of SERMs (DMA, FDMA, Ral; 1 µM). Protein expression of CYP1B1 and 1A1 was analyzed using western blot experiments as previously described (190). Anti-CYP450 1B1, anti-CYP450 1A1 and anti-β-actin antibodies were used as primary antibodies. Phase II detoxification enzymes were also analyzed using anti-SULT1, anti-SULT 1E1, anti-SULT 1A1, anti-GSTP1, anti-NQO1, and anti-COMT as primary antibodies. Antibodies were diluted in blocking solution (5% non fat milk in TBS with 0.1% tween 20). Blots were incubated with primary antibody overnight at 4 °C and with secondary antibody for 1 h at
room temperature. Blots were visualized using chemiluminescence substrate (Thermo scientific, Rockford, IL). Imaging and analysis was done using FluroChem software (Cell Biosciences, Santa Clara, CA). At least three independent western blot experiments were performed to confirm the effect on the enzymes.

4.2.5. Anchorage independent colony formation assay

Colony formation cell transformation assay was performed as previously described (190). Briefly, MCF-10A cells were treated with E$_2$ (1 µM) in the presence and absence of SERMs (1 µM) for 3 weeks, twice a week. At the end of the treatment, cells were seeded on soft agar (0.3% agar) at a density of 5 × 10$^4$ cells/well in 12 well plates precoated with 0.6% agar base medium. Estrogen free media was added as the feeding media on top of the soft agar layer. Cells were maintained in soft agar for 3 weeks and media was refreshed every 3 days. After 3 weeks, colonies were stained with crystal violet (0.005%) and analyzed using an Olympus inverted microscope (Center Valley, PA). Spherical formation of > 50 cells were taken as a colony. Number of colonies formed in each well were counted and represented as colony efficiency ± SD as previously described in chapter 2, materials and methods.

4.2.6. Detection of ROS in live cells

MCF-10A cells were grown (4 × 10$^3$ cells/mL) on each of eight chambers on a sterile NuncTM chambered coverglass and incubated overnight at 37 °C with 5% CO$_2$ in estrogen free media and formation of ROS was determined as previously described (193) with minor modifications. Cells were treated with E$_2$ (1 µM) with and without SERMs (1 µM) for 6 days. Treatments were renewed after 3 days. At the end of 6 days media was removed and cells were labeled with CM-H$_2$DCFDA (10 µM) for 30 min at 37 °C, 5% CO$_2$. Cells were rinsed with PBS to remove excess dye and nuclear was stained with 0.2 µg/mL Hoechst stain. Imaging of the cells was performed using Zeiss 510 META laser scanning confocal microscope.
4.2.7. Real time qPCR analysis of metabolizing enzyme gene transcripts

MCF-10A cell were plated at a density of $2 \times 10^5$ cells/well in a 6 well plate and treated with $E_2$ (1µM) with and without SERMs (1µM) for 24 h. Total RNA was isolated from cells using QIASHredder columns and QIAGEN RNeasy kit (Qiagen Inc., Valencia, CA) according to the manufacture protocol. Total RNA (1 µg) was used to synthesize cDNA using SuperScript III in a 20 µL reaction mixture according to manufacturer’s protocol. Real time qPCR analysis will be done with respective primers. TaqMan FAM probes and primers (AB Applied Biosystems, Foster City, CA) were used for the gene analysis of SULT 1A1, SULT 1E1 and UGT 1AI while human β-actin gene amplification was used as the internal control.

4.2.8. Detection and measurement of 8-oxo-dG

MCF-10A cells were plated in 15 cm diameter dishes at a density of $2 \times 10^6$ cells/dish in estrogen free media. Cells were allowed to attach for 1 day and then were treated with 4-OHE$_2$ (1 µM) with SERMs (1 µM) (DMA, Ral or F-DMA) and maintained until 80 -100% confluency for 72 h. 8-Oxo-dG analysis was done as described previously (184). The native dG was determined by HPLC (UV) scanning from 280 nm. The 8-oxo-dG was detected multiple reaction monitoring and collision-induced dissociation for the fragmentation pathway of $m/z$ 284 → 168 and $m/z$ 289 → 173 for [${^{15}N}_2]$8-oxo-dG using positive ion electrospray. The amount of 8-oxo-dG formed in respect to $10^6$ of dG was plotted against the respective treatment. Total 8-oxo-dG/ $10^6$ of dG ratio for the 4-OHE$_2$ treated sample was taken as 100% for the purpose of calculation.
4.3. Results

4.3.1. Effect of SERMs on estrogen metabolism

To evaluate the effect of SERMs on estrogen metabolism, the amount of 4-MeOE$_1$ and 2-MeOE$_1$ formed were measured using LC/MS-MS after treating the MCF-10A cells with E$_2$ (1 µM) in the presence or absence of SERMs (1 µM) for 6 days. Relatively higher amount of 2-MeOE$_1$ formation were consistently observed compared to 4-MeOE$_1$ in E$_2$ treated samples. There was an inhibition in 2- and 4-MeOE$_1$ formation with DMA and Ral treatment while a significant inhibition ($p < 0.05$) was observed for 4-MeOE$_1$ formation (Figure 24A) with both SERMs. Also there was a dose-dependent reduction in methyl ether formation with both DMA (Figure 24B) and Ral (Figure 24C). FDMA did not show any significant inhibition on the formation of either 2-MeOE$_1$ or 4-MeOE$_1$. 

Figure 24: (A) DMA and Ral significantly inhibit estrone methyl ether formation in MCF-10A cells while FDMA had no effect. MCF-10A cells were treated for 6 days with E₂ (1 μM) in the presence and absence of SERMs (1 μM). Cell media was analyzed for estrone methoxy ethers using LC/MS-MS. Amount of 2-MeOE₁ and 4-MeOE₁ formation in the cells were normalized to the internal standard. Estrone methyl ether formation in E₂ treated cells were taken as 100% and the other samples were graphed accordingly.
There was a significant inhibition in 4-MeOE₁ formation with the co-treatment of DMA and Ral, while FDMA had no effect. Each point represents an average of three independent experiments done in duplicates ± SD and the p values were calculated using ANOVA. (B), DMA and (C) Ral showed a dose dependent inhibition in both 4-MeOE₁ and 2-MeOE₁ formation in MCF-10A cells. Each point represents an average of two independent experiments performed in duplicates ± SD.

Figure 25: Modulation of estrone methyl ether formation by bezedoxifene (Baze) and 4-hydroxytamoxifen (4-OHTam). There was a significant reduction in the formation of 4-MeOE₁ with the treatment of Baze (1 μM), while 4-OHTam (1 μM) had no significant effect. MCF-10A cells were treated with E₂ (1 μM) in the presence and absence of SERMs (1 μM) for 6 days. Estrone methyl ether formation was analyzed using LC/MS-MS. Each point represents an average of two independent experiments performed in triplicates ± SD and p values were calculated using ANOVA.
Tamoxifen has been used in the prevention and treatment of ER(+) breast cancer (61). Tamoxifen is bioactivated to 4-hydroxytamoxifen (4-OHTam) in the presence of CYP450 enzymes (264). 4-OHTam is the active metabolite which is responsible for the biological activities of tamoxifen. 4-OHTam was also tested in the metabolism experiments with another clinical SERM bazedoxifene (Baze). There was no significant effect on the formation of estrone methyl ethers with 4-OHTam (1 μM) while a significant inhibition in 4-MeOE₁ formation was observed with the co-treatment of Baze (p < 0.05) (Figure 25).

To understand the effect of SERMs on major phase II conjugations, glucuronic acid groups and sulfate groups were hydrolyzed from estrogen metabolites. Cell media from the cells treated with E₂ in the presence and absence of SERMs for 6 days were collected and incubated with sulfatase and β-glucuronidase enzymes to hydrolyze off the sulfate and glucuronic acid conjugates. After hydrolysis and analysis of methyl ethers using LC/MS-MS, there was no significant difference in the amount of estrone methyl ether metabolites in E₂ treated and E₂ + SERM treated samples (Figure 26). The inhibitory effect of DMA and raloxifene was completely abolished with the hydrolysis of sulfate and glucuronic acid conjugates (Figure 26).
Figure 26: β-Glucuronidase and sulfatase hydrolyzed the phase II metabolites to reverse the effect of SERMs. MCF-10A cells were treated with E2 (1 μM) in the presence and absence of SERMs (1 μM) for 6 days. Cell media was collected and incubated with β glucuronidase and sulfatase at 37 °C overnight before measuring methoxy ether formation using LC/MS-MS. There was no significant effect in estrone methyl ether formation among any of the treatments. Each point represents an average of two independent experiments done in duplicate ± SD.

4.3.2. Effect of SERMs on ROS formation

To determine the effect of SERMs on E2 induced ROS formation, MCF-10A cells were incubated with E2 in the presence and absence of SERMs for 6 days and ROS were labeled with CM-H2DCFDA. Increased formation of ROS was detected with E2 treated samples compared to the vehicle control, DMSO. There was a decrease in E2 induced ROS formation when the cells were co-treated with DMA, Ral (Figure 27A), and Baze (Figure 27B). There was no significant effect on the formation of ROS with the co-treatment of FDMA (Figure 27A) and 4-OHTam (Figure 27B). ROS staining was evenly distributed in the cells in all the samples showing that ER mediated concentration of ROS into the nucleus is not prominent in this cell line.
Figure 27: (A) E$_2$ induced reactive oxygen species (ROS) formation in MCF-10A cells were inhibited by DMA and Ral, while FDMA had little effect. MCF-10A cells were treated with E$_2$ (1 μM) in the presence and absence of SERMs (1 μM) for 6 days and ROS were labeled with CM-H$_2$-DCFDA (10 μM) for 1 h. Nuclei were labeled with Hochest nuclear dye and DMSO (0.01%) treated cells were taken as the vehicle control. Live cells were imaged using confocal microscope META 510. Green color (Ex/Em; 488/530 nm) represent the DCF-DA while the nuclear is labeled in blue color (Ex/Em; 345/420 nm). (B) E$_2$ induced ROS formation in MCF-10A cells were attenuated with Baze co-treatment while 4-OHTam had no inhibitory effect.
4.3.3. Effect of SERMs on 8-oxo-dG formation

Measurement of the amount of 8-oxo-dG is a method to determine the level of oxidative DNA damage *in vitro* and *in vivo* (271). To determine the effect of estrogens on 8-oxo-dG formation, MCF-10A cells were treated with E$_2$ (1 μM) and 4-OHE$_2$ (1 μM) for 3 days. Since DMSO contributes to the formation of 8-oxo-dG to some extent there was always a higher background 8-oxo-dG in the control samples. Formation of 8-oxo-dG with E$_2$ (1 μM) was not significantly different from that of the DMSO control. Instead there was a significantly increased formation of 8-oxo-dG in the cells treated with 4-OHE$_2$ (1 μM) compared to the DMSO control (p < 0.001). Therefore to determine the effect of SERMs on estrogen induced 8-oxo-dG formation, MCF-10A cells were incubated with 4-OHE$_2$ (1 μM) and SERMs (1 μM) for 3 days and amount of 8-oxo-dG was analyzed using LC/MS-MS. There was a significant inhibition on 4-OHE$_2$ induced 8-oxo-dG formation with DMA and Ral (p < 0.05) while FDMA did not have any significant effect (*Figure 28*).

*Figure 28:* DMA and Ral significantly inhibit 4-OHE$_2$ induced 8-oxo-dG generation in MCF-10A cells while FDMA does not have any significant effect. MCF-10A cells were treated with 4-OHE$_2$ (1 μM) in the presence and absence of SERMs (1 μM) for 3 days and DNA was extracted and hydrolyzed to detect 8-oxo-dG formation using LC/MS-MS.
4.3.4. Effect of SERMs on malignant transformation

Upon exposure to carcinogenic compounds, MCF-10A and 10F cells could be transformed into a malignant phenotype (168, 204, 237). It has been shown previously that MCF-10A cells can get transformed into a malignant phenotype upon exposure to \( E_2 \) (1 µM) for 3 weeks and could form anchorage independent colonies in soft agar (190). To determine the effect of SERMs on \( E_2 \) induced malignant transformation, MCF-10A cells were treated with \( E_2 \) (1 µM) in the presence or absence of SERMs (1 µM) for 3 weeks followed by plating on semi-solid media. After three weeks on semi solid media, there was a significant inhibition on \( E_2 \) induced colony formation with DMA and Ral \((p < 0.05)\) while FDMA had no significant effect (Figure 29).

![Figure 29](image_url)

**Figure 29:** DMA and Ral significantly inhibit \( E_2 \) induced anchorage independent colony growth on soft agar while FDMA has little effect. MCF-10A cells were treated with \( E_2 \) (1 µM) in the presence and absence of SERMs (1 µM) for 3 weeks, twice a week. DMSO (0.01%) was used as the vehicle control while \( E_2 \) (1 µM) was used as the positive control. Cells were plated on soft agar and maintained in agar for 3 weeks. Percentage colony efficiency is calculated as the number of colonies formed per number of cells plated per well \( \times 100 \). Three independent experiments were done and the number of colonies formed in each well were counted and represented as percentage colony efficiency \( \pm SD \) and \( p \) values were calculated using ANOVA.
4.3.5. Effect of SERMs on enzyme expression

Cytochrome P450 1A1 and 1B1 enzymes act as phase I enzymes and are involved in the estrogen metabolism in breast epithelial tissues. To determine the effect of SERMs on phase I enzymes, MCF-10A cells were treated with E₂ (1 µM) in the presence or absence of SERMs (1 µM) for 6 days and protein expression of CYP450 enzymes were analyzed by immunoblotting. Although E₂ treatment increased the expression of CYP450 1A1 and 1B1, there was no significant effect on either of those enzymes expression with SERMs (Figure 30).

![Image of immunoblot analysis](image-url)

**Figure 30:** There was no significant effect on the CYP 1B1 and CYP 1A1 enzyme expression by any of the SERMs tested. MCF-10A cells were treated with E₂ (1 µM) in the presence and absence of SERMs (1 µM) for 6 days. Each lane contains 30 µg of total protein determined by BCA assay.

Detoxification enzymes play an important role in estrogen metabolism and facilitate the excretion of toxic metabolites by increasing the water solubility of the compounds. Among them SULT, UGT and GST play a major role. To determine the effect of SERMs on phase II enzymes, MCF-10A cells were treated with E₂ (1 µM) in the presence or absence of SERMs (1 µM) for 6 days and protein expression of phase II enzymes were analyzed by immunoblotting. There was a significant induction of SULT 1
enzyme expression with DMA and Ral co-treatment (Figure 31). There was an induction in SULT 1E1 enzyme expression with both DMA and Ral co-treatment (Figure 31). SULT 1A1 enzyme expression was not affected by any of the treatments (Figure 31). Yet, there was an induction in NQO1 enzyme expression with both DMA and raloxifene co-treatment (Figure 31). However, there was no effect on COMT and GSTpi (Figure 32) with any of the treatments.

![Figure 31: Effect of SERMs on phase II detoxification enzymes. There was an induction in SULT 1 enzymes with DMA and raloxifene co-treatment. There was an induction in SULT 1E1 with DMA and Ral while the effect on SULT 1A1 was minimal. There was an inhibition in NQO1 expression with E2 compared to the control, while DMA and Ral co-treatment induced NQO1. MCF-10A cells were treated with E2 (1 μM) in the presence and absence of SERMs (1 μM) for 6 days. Each lane contains 30 μg of total protein determined by BCA assay.](image-url)
Figure 32: There was no significant effect on GST pi and COMT enzyme expression with SERMs. MCF-10A cells were treated with E2 (1 μM) in the presence and absence of SERMs (1 μM). Protein lysates were prepared and expression of GST pi and COMT were analyzed using western blotting. Protein concentrations were determined by BCA assay and 30 μg of total protein was loaded into each lane.

4.3.6. Effect of SERMs on gene expression

Since there was a significant effect on detoxification enzymes with SERMs measured by immunoblotting, gene expression of those enzymes was determined using qPCR experiments to determine the expression at a different molecular level. Since there was an induction in the SULT 1 family protein expression with the co-treatment of DMA and raloxifene, qPCR experiments were performed to distinguish the SULT 1 family isozymes involved. E2 treatment increased the gene expression of SULT1E1 compared to the control sample. DMA and Ral co-treatments (p < 0.05) significantly increased the gene transcription of SULT1E1 compared to E2 treated samples, while the effect of FDMA was comparable to E2 sample (Figure 33A). There was an induction of SULT1A1 gene transcription with all the treatments including the E2 treatment (Figure 33C). There was no significant difference in the gene expression of SULT1A1 in E2 treated and SERM co treated samples. A significant effect (p < 0.05) was observed in UGT 1A1 gene expression with both DMA and Ral co-treatment compared to E2 treated sample, while there was no significant induction with FDMA (Figure 33B).
Although both *SULT1E1* and *UGT1A1* genes induced by E₂ treatment, significant induction was observed with *SULT1E1* gene induction with E₂ compared to the untreated control (*Figure 33*).

*Figure 33:* (A) Gene transcription of *SULT 1E1* was induced significantly by DMA and raloxifene while FDMA has no significant effect compared to control. (B) Gene transcription of *UGT 1A1* was induced significantly by DMA and and raloxifene. (C) There was an induction in *SULT1A1* gene with all the treatments. There was no significant difference between E₂ treated and E₂ + SERM treated samples with
respect to SULT1A1. Gene transcription was measured by qPCR after isolating RNA from 24 h treated MCF-10A cells. Each point represents an average of two independent experiments done in triplicates ± SD and the p values were calculated using ANOVA.
4.4. Discussion and conclusions

Selective estrogen receptor modulators (SERMs) have been used in the treatment and prevention of postmenopausal osteoporosis (272). Extensive studies have done for their effect on ER(+) breast cancer prevention and treatment, among which STAR (Study of Tamoxifen and Raloxifene) and IBIS (International Breast cancer Intervention Study) mainly focused on the primary prevention of breast cancer (273). There have been few studies on understanding the effect of SERMs on estrogen chemical carcinogenesis. The present study was designed to evaluate the effects of SERMs on oxidative E₂ metabolism and estrogen chemical carcinogenesis pathway in ER(-), non-tumorigenic, human breast epithelial cells (MCF-10A). We hypothesized that SERMs could modulate oxidative estrogen metabolism and chemical carcinogenesis in these cells.

Estrogen metabolism plays an important role in estrogen dependent cancer formation in hormone sensitive tissues. However, less is known about the correlation between SERMs and estrogen metabolism in breast epithelial cells. The present study demonstrates that SERMs can modulate E₂ induced malignant transformation by modulating oxidative estrogen metabolism in breast epithelial cells. Estrone methyl ether formation was significantly inhibited by the co-treatment of DMA and Ral while FDMA had no significant effect (Figure 24). Although it has been reported that the prototypical SERM, tamoxifen can attenuate estrogen metabolism in endometrial cells (274), there was no significant inhibition of estrone methyl ether formation with the active metabolite of tamoxifen, 4-OHTam in MCF-10A cells (Figure 25). However, a significant inhibition was observed with the co-treatment of another clinical SERM, Baze (Figure 25). This is the first report to show that there is an inhibitory effect with clinical and pre clinical SERMs on estrogen metabolism in breast epithelial cells.

Estrogen induced ROS formation is an indication of oxidative stress and possible genotoxicity leading to carcinogenesis (189). Exposure to E₂ metabolites showed an association with increased ROS formation in breast epithelial cells (89, 188). In the present study, it was observed that continuous exposure to E₂ for 6 days produced ROS in non-tumorigenic breast epithelial cells. There was a clear correlation with modulation of ROS formation and estrogen metabolism with all the clinical and pre-
clinical SERMs tested. It was reported that there is an increased generation of ROS with the catechol than with E$_2$, as the catechol can more rapidly participate in the redox reaction, especially in the presence of metal ions such as Cu$^{2+}$ and Fe$^{3+}$ (189, 275). Similarly, in the present study, when MCF-10A cell were treated with E$_2$ (1 μM) for 3 days formation of 8-oxo-dG was minimum and it was not significantly different from the DMSO control. Instead when the cells were treated with the catechol, 4-OHE$_2$ (1 μM) for 3 days there was an increase in 8-oxo-dG formation (Figure 28). There was a significant inhibition in the 4-OHE$_2$ induced 8-oxo-dG formation with Ral and DMA while FDMA had no significant effect (Figure 28). ROS formation, 8-oxo-dG formation, and oxidative DNA damage are interconnected cellular processes which can ultimately lead to genotoxicity and carcinogenesis (271, 276). Although there is an increased production of ROS with E$_2$ with the treatment of 6 days, it may not be enough to generate 8-oxo-dG in a detectable quantity in MCF-10A cells. Also, the rate of conversion of E$_2$ to 4-OHE$_2$ in the cells will affect the formation of ROS and 8-oxo-dG.

Malignant transformation can be a result of exposure of cells into carcinogenic/mutagenic chemicals, viruses and other agents causing genetic mutations. It has been shown previously that upon exposure to E$_2$ and its metabolites non tumerigenic breast epithelial MCF-10A cells can be transformed into a malignant phenotype which can form colonies in semi solid media (190, 237). In the present, study Ral and DMA could significantly inhibit E$_2$ induced malignant transformation in MCF-10A cells while FDMA had no effect (Figure 29). It has been reported that botanicals such as resveratrol and hops could inhibit E$_2$ induced malignant transformation in breast epithelial cells (125, 190). From the metabolism and ROS modulation data it could be postulated that 4-OHTam will not be able to inhibit E$_2$ induced malignant transformation, while Baze may have inhibitory effect. This can be used to explain in part as to why 4-OHTam has not been an effective on ER(-) breast cancer prevention and/or treatment.

Modulation of estrogen chemical carcinogenesis can be achieved via either inhibiting phase I metabolism to reduce the formation of catechols and quinones and/or via inducing phase II enzymes to increase the detoxification and elimination of toxic metabolites. It has been previously reported that hops and resveratrol can inhibit the expression of CYP450 1B1 in human breast epithelial cells to attenuate the
formation of estrogen catechols (190, 224). But in this study there was no significant effect of BT-SERMs on main cytochrome enzymes (CYP450 1A1 and CYP450 1B1) involved in extrahepatic E₂ metabolism (Figure 30). Phase II enzyme polymorphism and expression levels are associated with the risk of breast cancer in vitro, in vivo and in human subjects (143-145). Cytosolic sulfotransferases (SULTs) play a major role in hepatic as well as extra hepatic detoxification of xenobiotics and other toxic metabolites (105). It has been reported that SULT 1E1 and SULT 2B1 are responsible for the sulfation of estrogenic catechols (277). There are evidence that SULT 1E1 and SULT2B1 are highly expressed in MCF-10A cells compared to breast cancer cells such as T47D, SKBR3 and MDA-MB-231 (277). Growing body of evidence support that there is an association between breast cancer and genetic polymorphism in human SULT1A1 and UGT1A1 genes (278). The expressions of these enzymes were analyzed with and without E₂ and SERM treatment in MCF-10A cells. It turned out that SULT 1 family was induced by both DMA and Ral co-treatment (Figure 31). With q-PCR experiments done with SULT 1 family isozyme primers (1A1, 1E1, 2B1), it was observed that SULT 1E1 was induced by DMA and Ral while SULT 1A1 and 2B1 were not affected. Among the other detoxification enzymes tested UGT 1A1 was induced with DMA and Ral treatment but the expression of UGT 1A1 was very low in MCF-10A cells to be tested by immunoblotting. It can be postulated that the induced of SULT 1E1 with DMA and Ral can be one of the major mechanisms to modulate estrogen metabolism in MCF-10A cells.

SERMs can get bioactivation by cytochrome P450 enzymes at physiological conditions to form reactive metabolites such as quinones and quinone methides (279, 280). In the present study BT-SERMs which can form quinoids (DMA, Ral) (280, 281) could inhibit estrogen metabolism, while those which cannot form a stable quinoid (FDMA) (280) could not inhibit the oxidative estrogen metabolism. Also it is chemically feasible to Baze to get bio activated to a quinoid. In the present study Baze but not 4-OHTam could inhibit estrone methyl ether formation in MCF-10A cells. So it could be possible that the mechanism behind the inhibitory effect of these SERMs mediated via formation of di-quinone methide (quinoid) in the physiological conditions. SERMs modulate the activity of estrogens by binding to ER in ER(+) cells (273). Since MCF-10A cells are ER α (-) mechanism of actions of SERMs may be exerted via
either ER β, Aryl hydrocarbon receptor or another orphan receptor which can get activated by SERM ligands. Further studies should be done to investigate the receptors and signal transduction pathways involved in the mechanism of action of SERMs in ER(-) cells.

Figure 34: Possible mechanism of action of SERMs act as inhibitors of estrogen carcinogenesis in human breast epithelial cells. E2 induces both CYP450 1B1 and 1A1 while there was no effect by SERMs on CYP450 enzymes. SERMs attenuate oxidative estrogen metabolism by inducing phase II detoxification enzyme such as, sulfotransferase (SULT), UDP glucuronic acid transferase (UGT), NAPDH dependent quinone reductase (NQO1) and glutathione-S-transferase (GST).
In conclusion, there was a significant inhibition in oxidative estrogen metabolism, E₂ induced ROS generation and E₂ induced malignant transformation with DMA and Ral in human breast epithelial cells. Also there was a significant inhibition in estrone methyl ether formation and E₂ induced ROS formation with Baze while 4-OHTam didn’t have any effect. This study presents the novel concept of SERMs acting as E₂ chemical carcinogenesis pathway modulators by attenuating oxidative estrogen metabolism. The present study may lead SERM research into a new direction to investigate the potential of SERMs to attenuate ER independent estrogen carcinogenesis. It is demonstrated in the present study that clinical and pre clinical SERMs have the potential to act as breast cancer chemopreventive agents in ER independent manner in addition to their well known ER dependent pathway. Further studies should be designed to understand the molecular targets and receptors that SERMs act on in ER(-) cells to modulate estrogen carcinogenesis. Additional studies should be done to investigate the effects of these SERMs in animal models in order to understand the pharmacokinetics and bioavailability.
5: PREVENTION OF ESTROGEN CARCINOGENESIS BY MODULATING NO/CGMP PATHWAY

5.1. Introduction

5.1.1. Nitric oxide (NO); Biosynthesis and physiological role

Nitric Oxide (NO) is a gaseous free radical soluble in water as well as in lipids. Due to the solubility in lipids it makes NO a better inter and intra cellular signaling molecule (81). NO is important in many physiological and pathological processes and was named as the “molecule of the year” in 1992 by Science journal (282). It was first reported by Palmer et.al that L-arginine is the precursor for the biosynthesis of NO in endothelial cells (283). The reaction of NO biosynthesis from L-arginine is catalyzed by the enzyme nitric oxide synthase (NOS) (Figure 35) in the presence of many other cofactors (284). Three isoforms of NOS have been identified; viz, neuronal NOS (nNOS) (NOS I), inducible NOS in macrophages (iNOS) (NOS II) and endothelial NOS (eNOS) (NOS III). These isoforms have named depending on the tissues they were originally extracted and frequently more than one isoform is available in one tissue type (285). All NOSs are fully active as homodimers and many cofactors are needed to catalyze this reaction (83). The eNOS and nNOS are considered constitutively expressed enzymes while iNOS is inducible (83).

\[
\text{L-Arginine} + \text{O}_2 + \text{H}_2\text{O} + \text{NADPH} + \text{H}^+ \rightarrow \text{L-Citrulline} + \text{NO} \cdot + \text{NADP}^+ 
\]

*Figure 35: Biosynthesis of NO from L-arginine is catalyzed by NOS.*
The importance of NO in cardiovascular system, immune system and central nervous system has been extensively studied (83, 286, 287). In cardiovascular system NO is mainly produced in endothelium by eNOS and responsible in the regulation of blood pressure and vascular tone, inhibition of platelet aggregation and leukocyte adhesion and prevention smooth muscle cell proliferation (287). Reduced bioavailability of NO is one of the main causes of occurrence of cardiovascular diseases such as hypertension, atherosclerosis and heart failure (287). It has been reported that NO can act as a neurotransmitter at least in some of the neurons which are known as “nitregic nerves” (288). There has been reports to show the positive correlation between increased NO production and the occurrence of neurological diseases such as Alzheimer’s and Parkinson’s diseases (289). NO has a role as a bacteriocide when released in large quantities by activated macrophages (290).

Nitric oxide can closely interact with iron containing proteins and binds to heme. Due to the high affinity of NO in binding to heme group, soluble guanylate cyclase (sGC) could get activated via binding to the heme group (83). Upon binding of NO to sGC, it could catalyze the formation of cyclic GMP (cGMP) from the nucleotide GTP. Many of the physiological actions of NO are put forth by the activation of this sGC/cGMP pathway (291).
5.1.2. NO/cGMP signaling pathway and cancer

Though the connection of NO and cancer is not well understood, there are reports to support the fact that NO is important both positively and negatively in tumor formation (85). All the three isoforms of NOS have been detected in tumor cells and they are involved in either promoting or inhibiting cancer formation (85). The biological effects of NO which can lead to the formation of cancer, depends on the oxidative metabolism of NO, differential expression of NOS, and the concentration of NO in the tissue. Oxidative metabolism of NO can lead to the generation of peroxynitrite ion (ONOO\(^-\)), N\(_2\)O\(_3\), and other reactive nitrogen species which can cause DNA damage and progression of cancer (292). Oxidative metabolism of NO can lead to DNA damage and mutations in human lymphoblastoid cells (293).

Thomsen et.al showed that there is increased NO biosynthesis in invasive breast tumors compared to the benign tissue (86). In the same study, higher NO biosynthesis in grade III breast tumors was reported compared to grade II tumors suggesting that increased NOS activity and/or expression could be correlated with cancer (86). Also growing body of evidence shows that eNOS inhibits apoptosis,
promotes angiogenesis, cancer cell proliferation, invasion, and metastasis (82, 294, 295). Additionally, poor prognosis was observed in ER(-) breast cancer patients with an increased expression of iNOS in the tumor (296). Also, a study done by Lee et. al showed that there is a correlation between polymorphisms of NOS and aggressive prostate cancer (297). Although the mechanism of action is not clearly understood, these studies collectively show that there is a correlation between NOS and carcinogenesis.

Few studies have done to evaluate the correlation between estrogen carcinogenesis and NO. It has been shown that estradiol could stimulate constitutive NOS in breast tissue and the NOS expression was strongly correlated with ER expression in these cells (298). Also, NO production can be induced by estrogen in human neuroblastoma cells via activating constitutive NOS (299).

The present study was designed to evaluate the effect of NO modulation on estrogen carcinogenesis in ER(-) breast epithelial cells. It was hypothesized that inhibition of NO production could attenuate oxidative estrogen metabolism and E₂ induced malignant transformation in these cells.
5.2. Materials and methods

5.2.1. Cell lines and cell culture conditions

MCF-10A cells were used for all the experiments and the cell culture conditions were maintained as previously described in chapter 2, materials and methods.

5.2.2. Chemicals and reagents

All the chemicals and reagents were obtained from Sigma (St. Louis, MO), Invitrogen (Carlsbad, CA) or Cayman Chemical (Ann Arber, MI) unless stated otherwise.

Figure 37: Chemical structures of compounds used in the study.

5.2.3. Analysis of estrogen metabolites

MCF-10A cells were incubated with E\textsubscript{2} (1 μM) with and without L-NAME (5 μM) for 6 days and the metabolites were analyzed using LC/MS-MS as previously described in Chapter 2, materials and methods. Since there was a significant inhibition in the formation of methyl ethers with the co- treatment
of the NOS inhibitor; L-NAME further studies were done to modulate NO/cGMP pathway and to evaluate the effect on estrogen metabolism. ODQ (NO-GC inhibitor, 1 μM) and KT-5823 (PKG inhibitor, 1 μM) used as the pathway inhibitors and cells were incubated with these compounds in the presence of E₂ (1 μM) for 6 days and the metabolites were analyzed using LC/MS-MS.

Further experiments were done to determine whether there is any additive or synergistic inhibitory effect with NOS inhibition and SERMs. MCF-10A cells were treated with E₂ (1 μM) in the presence and absence of L-NAME (5 μM) and DMA (1 μM) for 6 days and estrone methyl ether formation was analyzed using LC/MS-MS as previously described (190).

5.2.4. Detection of ROS in live cells

Since there was a significant effect on estrogen methyl ether formation with L-NAME treatment, its effect on E₂ induced ROS generation was also analyzed. MCF-10A cells were incubated with E₂ (1 μM) with and without L-NAME (5 μM) for 6 days and the formation of ROS in live cells were imaged using confocal microscope as previously described in chapter 2, materials and methods.

5.2.5. Anchorage independent colony formation assay

The effect of L-NAME on E₂ induced malignant transformation was analyzed by anchorage independent colony formation assay. MCF-10A cells were incubated with E₂ (1 μM) with and without L-NAME (5 μM) for 3 weeks and cells were plated on agar for 3 weeks. Anchorage independent colony formation was evaluated as previously described in chapter 2, materials and methods.

5.2.6. Immunoblotting

MCF-10A cells were treated with E₂ (1 μM) in the presence and absence of L-NAMEs (5 μM). Protein expression of CYP1B1 was analyzed using western blot experiments as previously described (190). Anti-CYP450 1B1 and anti-β-actin antibodies were used as primary antibodies. Antibodies were diluted in blocking solution (5% non fat milk in TBS with 0.1% tween 20). Blots were incubated with primary antibody overnight at 4 °C and with secondary antibody for 1 h at room temperature. Blots were
visualized using chemiluminescence substrate (Thermo scientific, Rockford, IL). Imaging and analysis was done using FluroChem software (Cell Biosciences, Santa Clara, CA).
5.3 Results

5.3.1. Effect of NOS inhibitors on estrogen metabolism

When the cells were treated with L-NAME (5 μM) and estrogen methyl ethers were analyzed using LC/MS-MS, there was a significant inhibition in the formation of 4-MeOE₁ (Figure 38). However, there was no significant inhibition in the formation of 2-MeOE₁ with the co-treatment of L-NAME (Figure 38).

Figure 38: L-NAME significantly inhibited the formation of 4-MeOE₁ in MCF-10A cells. Cells were treated with E₂ (1 μM) and E₂ (1 μM) + L-NAME (5 μM) for 6 days. Cell media was collected and analyzed for estrone methyl ether formation. Each data point represents an average of two independent experiments done in duplicate ± SD and the p values were calculated using ANOVA.
5.3.2. Effect of NO/cGMP pathway modulators on estrone methyl ether formation

Since there was a significant inhibition in the formation of 4-MeOE$_1$ with the NOS inhibitor, L-NAME, further experiments were done to evaluate the effect of NO/cGMP pathway modulators on estrogen metabolism. Soluble Guanylate cyclase inhibitor ODQ (5 μM) and protein kinase G inhibitor KT5823 (1 μM) showed a significant inhibition in estrone methyl ether formation in MCF-10A cells. However, no selectivity towards 4-MeOE$_1$ was observed with the treatment of ODQ (5 μM) and KT5823 (1 μM), instead both 2- and 4-MeOE$_1$ were reduced (Figure 39).

![Figure 39: ODQ (5 μM) (sGC inhibitor) and KT-5823 (1 μM) (PKG inhibitor) significantly inhibited the formation of methyl ether metabolite in MCF-10A cells. Treatments were done for 6 days and estrone methyl ether formation was analyzed with LC-MS/MS. Each data point represents an average of three independent experiments done in duplicates ± SD and the p values were calculated using ANOVA * p < 0.05.,](image-url)
5.3.3. Additive effect of DMA and L-NAME together on estrogen methyl ether formation.

Figure 40: There was an additive effect on the formation of methyl ether metabolites with the treatment of DMA and L-NAME in MCF-10A cells. Treatments were done as indicated in the figure (DMA, 1 μM; L-NAME, 5 μM) for 6 days followed by extraction of cell media for estrogen metabolites. Amount of estrone methyl ether formation was analyzed with LC/MS-MS. Each data point represents an average of two independent experiments done in duplicates ± SD and p values were calculated by ANOVA.

It was observed previously that DMA at 1 μM could inhibit estrone methyl ether formation in MCF-10A cells (Figure 24). Also there was an inhibitory effect on the formation of estrone methyl ether with L-NAME 5 μM (Figure 38). Further experiments were done to find out whether there is any synergistic or additive effect on estrone methyl ether formation with these two compounds in MCF-10A cells. When MCF-10A cells were treated with E2 in the presence and absence DMA + L-NAME, there was a significant effect on estrone methyl ether formation (Figure 40). However, no synergic effect was observed with DMA + L-NAME treatment and instead an additive inhibitory effect was observed in the
formation of methyl ethers. In all the treatment groups there was a selectivity towards the inhibition of 4-MeOE₁ over 2-MeOE₁.

**5.3.4. Effect of L-NAME on E₂ induced ROS formation in MCF-10A cells.**

When the cells were treated with E₂ and E₂ + L-NAME for 6 days and analyzed for ROS generation using confocal microscope, there was an inhibition in E₂ induced ROS formation in L-NAME co treated samples (*Figure 4I*).

*Figure 4I*: Representative images showing the effect of L-NAME on E₂ induced ROS formation in MCF-10A cells. There was an inhibition in the E₂ induced ROS formation with the co-treatment of L-NAME (5 μM). MCF-10A cells were treated with E₂ in the presence and absence of L-NAME for 6 days and ROS was labeled with CM-H₂DCFDA (10 μM) for 1 h. Nuclei were labeled with Hoechst nuclear dye and DMSO (0.01%) treated cells were taken as the vehicle control. Live cells were imaged using confocal microscope META 510. Green color (Ex/Em; 488/530 nm) represent the DCF-DA while the nuclear is labeled in blue color (Ex/Em; 345/420 nm).
5.3.5. Effect of NO donors on estrone methyl ether formation in MCF-10A cells.

Since there was a significant inhibition in estrone methyl ether formation with NOS inhibition, further experiments were done to evaluate whether exogenous NO can counteract the effect of NOS inhibition. In order to demonstrate the effect of exogenous NO on the estrone methyl ether formation, two well known NO donors (DETANO, Cys-NO) were used in the metabolism experiments. MCF-10A cells were treated with E₂ in the presence and absence of L-NAME (5 μM), L-NAME (5 μM) + DETANO (5 μM) and L-NAME (5 μM) + Cys-NO (5 μM) for 6 days and cell media was analyzed for estrone methyl ether formation. There was a significant inhibition with the treatment of L-NAME in the methyl ether formation and the inhibitory effect was not affect by the treatment of NO donors (Figure 42).

Figure 42: Representative chromatograms showing the effect of NO donors on methyl ether formation. There was a significant inhibition of methyl ether formation with the treatment of L-NAME (5 μM) (blue line). Neither DETNO (5 μM) (green line) nor Cys NO (5 μM) (red line) could reverse the effect of L-NAME, instead they help to further reduce the amount of methyl ether formation but not in a significant amount. MCF-10A cells were treated with indicated compounds for 6 days and cell media was analyzed for estrone methyl ether formation using LC/MS-MS.
5.3.6. Effect of L-NAME on malignant transformation.

In order to find out the effect of L-NAME on E<sub>2</sub> malignant transformation, anchorage independent colony formation was analyzed with the treatment of E<sub>2</sub> in the presence and absence of L-NAME. DMSO was used as the vehicle control. There was a significant inhibition (p < 0.0001) in E<sub>2</sub> induced malignant transformation with L-NAME (Figure 43).

Figure 43: L-NAME significantly inhibited (p < 0.001) E<sub>2</sub> induced malignant transformation in MCF-10A cells. Cells were treated with E<sub>2</sub> (1 μM) in the presence and absence of L-NAME (5 μM) for 3 weeks. At the end of 3 weeks, cells were plated and maintained on soft agar for 3 weeks and formation of colonies were observed and counted using an inverted microscope. DMSO treated (0.01%) samples were used as a negative control. Each point represents an average of triplicate ± SD and the p values were calculated by ANOVA.
5.3.7. Effect of L-NAME on CYP450 1B1 expression.

MCF-10A cells were incubated with E₂ (1 μM) in the presence and absence of L-NAME (5 μM) for 6 days and protein expression of CYP450 1B1 was analyzed. There was no significant effect on the expression of CYP450 1B1 with the L-NAME treatment (Figure 44).

Figure 44: Effect of L-NAME on the expression of CYP450 1B1 in MCF-10A cells. There was no significant effect on the CYP450 1B1 expression with the treatment of L-NAME (5 μM). MCF-10A cells were treated with E₂ (1 μM) in the presence and absence of L-NAME for 6 days and protein expression of CYP450 1B1 was analyzed with western blotting. Each lane contains 30 μg of total protein.
5.4. Discussion and conclusions

Nitric oxide is a free radical cellular messenger molecule important in many physiological and pathological processes. The correlation between estrogen dependent cancer and NO production has been reported previously (87, 299, 300). The present study was done to evaluate the effects of NO inhibition on oxidative estrogen metabolism and estrogen carcinogenesis. It was hypothesized that inhibition of NO production could influence the attenuation of estrogen carcinogenesis via modulating oxidative estrogen metabolism. The hypothesis was proven to be correct and it was observed that the NOS inhibitor L-NAME was able to modulate estrogen metabolism, E2 induced ROS formation, and E2 induced malignant transformation in MCF-10A cells.

Long term exposure to endogenous estrogens either through early puberty and/or late menopause can increase the risk of developing estrogen dependent cancer (26, 301). The mechanism of estrogen dependent cancer formation depends on ER mediate increased cell proliferation and/or production of highly reactive estrogen metabolites (27, 95, 302). Estrogens can get metabolized to 2- and 4-hydroxycatechols in the presence of CYP450 1A1 and 1B1, respectively in breast epithelium (303). Both catechols can get further oxidized to highly reactive quinones while 4-hydroxycatechol is considered more genotoxic (97, 101). Researchers have tried to find a correlation between NO production and estrogen carcinogenesis (85, 87, 296, 298). E2 can induce NOS and increased the production of NO in an ER dependent and independent manner (304). Also there have been reports to show that increased production of NO is associated with increased cancer risk (85, 86). In the present study, experiments were designed to understand the effects of NOS inhibition on oxidative estrogen metabolism in MCF-10A cells. It was observed that L-NAME; a non selective NOS inhibitor, could inhibit estrone methyl ether formation in MCF-10A cells. There was a significant inhibition (p < 0.005) on the formation of 4-MeOE1 by L-NAME (5 μM) while no significant inhibition was observed in the formation of 2-MeOE1 (Figure 38). Since 4-hydroxylation is considered more genotoxic, it is important that the compounds can specifically inhibit 4- hydroxylation while 2-hydroxylation is not affected. This is the first report to show the correlation between NO and estrogen metabolism in breast epithelial cells. Since NO mediates most of
its biological actions via NO/sGC/cGMP pathway, inhibitors for this pathway was used to evaluate the importance of this pathway in estrogen metabolism. When the MCF-10A cells were treated with sGC inhibitor ODQ and protein kinase G inhibitor KT5823, there was a significant inhibition in the formation of estrone methyl ether (Figure 39). However, there was no selectively for inhibition of 4-hydroxylation with either ODQ or KT5823. In order to find out whether exogenous NO can counteract the effects of L-NAME, MCF-10A cells were treated with L-NAME and NO donors before analyzing estrone methyl ethers. There was no significant effect observed with the treatment of NO donors and they could not reserve the effect of L-NAME on estrogen metabolism in MCF-10A cells (Figure 42) suggesting that exogenous NO can not counteract the effect of L-NAME.

Increased ROS production is indicative of oxidative stress and possibly carcinogenesis (205, 305). In the present study, it was observed that when NOS was inhibited with L-NAME, there was a reduction in the E2 induced ROS formation in MCF-10A cells (Figure 41). NOS could also contribute to the production of superoxide anion and hydrogen peroxide apart from biosynthesis of NO (306). Therefore, the observed inhibitory effect on ROS formation by L-NAME could be due to inhibition of oxidative estrogen metabolism and/or by inhibition of synthesis of ROS from NOS.

MCF-10 cells can be transformed into a malignant phenotype upon exposure to carcinogens such as TCDD, E2, or E2 metabolites (125, 169, 190, 237). It has been reported that NO can mediate malignant transformation of human lung epithelial cells by stabilizing the apoptotic pathway component, bcl-2 (307). Contradictory results were published from a study done with MCF-7 and MDA-MB-231 cells, which showed that NO could decrease the motility and increase adhesion of human breast cancer cells, and thus the aggressiveness was increased with the loss of NOS (308). However, in the present study, it was observed that NOS inhibition by L-NAME, attenuated E2 induced malignant transformation and anchorage independent colony formation of MCF-10A cells (Figure 43). There are reports to show that malignant transformation of MCF-10A cells are mediated by induction of NF-κB pathway (237). Also there is a correlation between NO and NF-κB pathway (309). A possible mechanism of inhibition of malignant transformation could be through inhibition of NF-κB pathway in MCF-10A cells. Further
studies should be done to understand the molecular mechanism of L-NAME acting as an inhibitor of malignant transformation in MCF-10A cells.

Previously it was observed that DMA (Figure 24) and L-NAME (Figure 38) could significantly inhibit the formation of estrone methyl ethers in MCF-10A cells. In order to find out whether there is any synergistic effect on estrogen metabolism, MCF-10A cells were co-treated with L-NAME and DMA, followed by the analysis of estrone methyl ethers. No synergistic effect was observed when the cells were co-treated with L-NAME and DMA (Figure 40), instead there was an additive effect (Figure 40). It was previously mentioned that DMA inhibits estrogen metabolism via inducing phase II conjugative metabolic enzyme expression. As for DMA, there was no significant effect on the expression of CYP450 1B1 with L-NAME treatment (Figure 44). In this case L-NAME also could be modulating phase II conjugative metabolism since there was no synergistic effect with co-incubation.

In conclusion, there was a significant inhibition in oxidative estrogen metabolism, E2 induced ROS formation, and malignant transformation with the treatment of NOS inhibitor, L-NAME in human breast epithelial cells. Also there was a significant inhibition in estrone methyl ether formation with the treatment of sGC/cGMP pathway modulators. This is the first study to show that there is a correlation between NO and estrogen metabolism and estrogen carcinogenesis in human breast epithelial cells. This study should be continued to further to understand the mechanism of action of L-NAME and to determine the molecular targets and receptors involved.
6. CONCLUSIONS AND FUTURE DIRECTIONS

Mechanism of estrogen chemical carcinogenesis and the possible biomarkers in estrogen carcinogenesis has been studied in the present study in order to broaden the understanding about estrogen related cancer formation. Measurement of estrone methyl ethers, anchorage independent colony formation and estrogen induced ROS formation could be used as useful biomarkers in the process of assessment of estrogen carcinogenicity in vitro. Potential breast cancer chemopreventive activity of a compound can be estimated by the ability to attenuate oxidative estrogen metabolism, estrogen induced malignant transformation and ROS formation in breast epithelial cells. In the present study it is hypothesized that botanicals, SERMs and NO/cGMP pathway modulators could attenuate oxidative estrogen metabolism and estrogen induced malignant transformation in human breast epithelial cells and could act as breast cancer chemopreventive agents. This hypothesis was proven to be true for hops, DMA, Ral and L-NAME among the compounds and botanical extracts tested.

From the two botanicals tested, hops showed a significant inhibition on estrone methyl ether formation while black cohosh had no effect. Hops mediated this inhibitory effect via inhibiting the estrogen induced upregulation of CYP450 1B1 and CYP450 1A1. The hops compounds, XH and 8-PN showed a significant inhibition in estrogen induced anchorage independent colony formation although only 8-PN had a significant inhibitory effect on estrogen metabolism. XH and 8-PN could be acting via two different pathways to attenuate estrogen induced malignant transformation in MCF-10A cells. Although 8-PN possesses potent estrogenic activites in endometrial cells it showed an inhibitory activity towards estrogen induced malignant transformation in breast epithelial cells, suggesting that 8-PN could be behaving as a SERM.

Present study evaluated the effects of clinical and pre clinical SERMs on the oxidative estrogen metabolism and estrogen induced malignant transformation in breast epithelial cells. It can be concluded that DMA, Ral and Baze showed a significant inhibition on estrone methyl ether formation and estrogen
induced ROS formation while FDMA and 4-OHTam had little effect. DMA and Ral attenuated estrogen metabolism via inducing phase II conjugative enzyme expression in MCF-10A cells. The breast cancer preventive abilities of Ral have been extensively evaluated in pre clinical and clinical studies, yet the main focus was given to ER (+) breast cancer. For the first time in the present study Ral was tested in ER (-) breast epithelial cells in order to understand the effect of Ral on the estrogen chemical carcinogenesis pathway. It was observed that both Ral and DMA act as potent inhibitors towards the estrogen induced malignant transformation in this cell line. Results of the present study can be used for the advancement of the knowledge of SERMs and to understand SERM action in an ER independent cellular system.

Attenuation of NO/cGMP signaling pathway resulted in significant inhibition of oxidative estrogen metabolism and estrogen induced malignant transformation in breast epithelial cells. This is the first report to show the association between the NO/cGMP pathway and estrogen chemical carcinogenesis pathway.

This study can contribute to the improvement of understanding in the fields of botanicals, SERMs and NO chemistry. It is essential to rule out the effect of hormonal pathway in the metabolism studies and further experiment should be done with an ER antagonist (ICI) to find the effect on estrogen metabolism and estrogen induced malignant transformation. Further studies will be designed to discover the molecular targets and possible receptors that SERMs, hops and L-NAME acting on to exert the inhibitory effects towards estrogen carcinogenesis. Also future studies should be designed to evaluate the effects of hops, 8-PN, DMA, Ral, and L-NAME in animal models.
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EDUCATION

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• 3rd Place in oral presentation, 50th annual MIKI meeting held at University of Iowa - 2012
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Title: “Selective estrogen receptor modulators (SERMs) act as breast cancer chemopreventive agents in human mammary epithelial cells by attenuating oxidative estrogen metabolism.”
2011- Oral presentation at College of Pharmacy, UIC in September 2011
Title: “Molecular mechanism of breast cancer chemoprevention”
2011- Presented a scientific poster at ACS national meeting held in Denver, CO in August 2011
Title: “Selective estrogen receptor modulators inhibit oxidative stress and malignant transformation in breast epithelial (MCF-10A) cells”.
2011- Presented a poster at the Society of Toxicology (SOT) meeting held in Washington DC, in March 2011
Title: “Hops (Humulus lupulus) inhibits estrogen metabolism in non-tumorigenic human breast epithelial cells (MCF-10A)”
2010- Presented a scientific poster at the great lakes drug metabolism discussion group meeting held in Saint Louis, MO, in May 2010.
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