Hops (Humulus lupulus) inhibits Oxidative Estrogen Metabolism and Estrogen-Induced Malignant Transformation in Human Mammary Epithelial cells (MCF-10A)

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Abstract

Long-term exposure to estrogens including those in traditional hormone replacement therapy (HRT) increases the risk of developing hormone-dependent cancers. As a result, women are turning to over-the-counter (OTC) botanical dietary supplements such as black cohosh (Cimicifuga racemosa) and hops (Humulus lupulus) as natural alternatives to HRT. The two major mechanisms which likely contribute to estrogen and/or HRT cancer risk are: the estrogen receptor (ER) mediated hormonal pathway; and, the chemical carcinogenesis pathway involving formation of estrogen quinones that damage DNA and proteins, hence initiating and promoting carcinogenesis. Since OTC botanical HRT alternatives are in widespread use they may have the potential for chemopreventive effects on estrogen carcinogenic pathways in vivo. Therefore the effect of OTC botanicals on estrogen-induced malignant transformation of MCF-10A cells was studied. Cytochrome P450 catalyzed hydroxylation of estradiol at the 4-position leads to an o-quinone believed to act as the proximal carcinogen. LC-MS/MS analysis of estradiol metabolites showed that 4-hydroxylation was inhibited by hops, whereas black cohosh was without effect. Estrogen-induced expression of CYP450 1B1 and CYP450 1A1 was attenuated by the hops extract. Two phenolic constituents of hops (xanthohumol, XH; and 8-prenylnaringenin, 8-PN) were tested: 8-PN was a potent inhibitor whereas XH had no effect. Finally, estrogen-induced malignant transformation of MCF-10A cells was observed to be significantly inhibited by hops (5 µg/mL) and 8-PN (50 nM). These data suggest that hops extracts possess cancer chemopreventive activity through attenuation of estrogen metabolism mediated by 8-PN.
Footnotes

\(^1\)Abbreviations: AhR, Aryl hydrocarbon receptor; E\(_2\), 17\(\beta\)-estradiol; COMT, catechol O-methyl transferase; ER, estrogen receptor; HRT, hormone replacement therapy; 2-OHE\(_1\), 2-hydroxyestrone; 4-OHE\(_1\), 4-hydroxyestrone; 2-OHE\(_2\), 2-hydroxyestradiol; 4-OHE\(_2\), 4-hydroxyestradiol; LC-MS/MS, liquid chromatography-tandem mass spectrometry; OTC, over-the-counter; 2-MeOE\(_1\), 2-methoxyestrone; 4-MeOE\(_1\), 4-methoxyestrone; 2-MeOE\(_2\), 2-methoxyestradiol, 4-MeOE\(_2\), 4-methoxyestradiol; 8-PN, 8-prenylnaringenin; SERMs, selective estrogen receptor modulators; XH, xanthohumol
Introduction

Long-term exposure to estrogen resulting from a combination of early onset of menstruation, nulliparity or delayed first child birth, short duration of breast feeding, late menopause, and use of hormone replacement therapy (HRT) (1, 2) increases the risk of hormone-dependent cancers in women (3, 4). Two major mechanisms for estrogen carcinogenesis have been proposed which include estrogen-induced cell proliferation in estrogen receptor (ER) positive cells (hormonal pathway) and the formation of reactive estrogen metabolites (chemical pathway, Fig. 1) (5). Understanding these mechanisms can lead to strategies for prevention of estrogen-dependent cancers which can enhance the quality of life for women as well as significantly reduce the cost of health care.

Modulation of the hormonal mechanism has had some success with selective estrogen receptor modulators (SERMs) and aromatase inhibitors (6-9). Reduction in estrogen cancer risk could also be achieved through modulation of enzymes involved in generation of reactive estrogen metabolites (chemical pathway, Fig. 1). In breast epithelium, 17β-estradiol (E2) can be converted to its 2-hydroxy (2-OHE2) and 4-hydroxy (4-OHE2) catechols catalyzed by CYP450 1A1/1A2 and CYP450 1B1, respectively (Fig. 1). Both catechols are further oxidized to form reactive electrophilic o-quinones which have the potential to cause DNA and protein damage leading to carcinogenesis (5). Several detoxification pathways can potentially neutralize catechols and quinones in cells; for example, catechol O-methyl transferase (COMT) converts catechols to methyl ether metabolites that cannot form an o-quinone (10) (Fig. 1). The expression of these enzymes differs from one tissue to the other and can be altered when a normal cell is transformed into a cancer cell, leading to an imbalance in estrogen metabolism (11, 12). Agents that modulate enzyme activity through the inhibition or regulation of transcription are expected to have chemopreventive properties, provided these agents attenuate estrogen quinone formation (3, 4).
The MCF-10A cell line is a non-tumorigenic, immortalized human breast epithelial cell line, which is classified as ERα negative because estrogens do not induce proliferation (13). The latter attribute makes this a useful model system for study of chemical carcinogenesis as modulation of estrogen metabolism can be clearly demonstrated in this system without interference from hormonal carcinogenic pathways. In addition, MCF-10A cells can be transformed into a malignant phenotype by estrogenic compounds including E2 and equine estrogens (14, 15). More importantly, it has been reported that OTC botanical dietary supplements, such as resveratrol, can modulate the estrogen-induced malignant transformation and CYP450 enzyme expression in MCF-10A cells (16) and in the related MCF-10F cell line (17).

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 (18, 19). Their mechanisms of action are not completely known; however, estrogenic activity for hops (20-22) and serotonergic effects for black cohosh (23) has been reported. Hops, commonly used as a flavoring agent in beer, has been used as a dietary supplement for sleep disturbances and in the treatment of mood disorders (24). Hops contain numerous active ingredients, including xanthohumol (XH) that has demonstrated chemopreventive activity (24-27). Black cohosh extracts have been reported to inhibit breast cancer cell growth both in ER positive and negative cell lines (28, 29). We hypothesized that hops and black cohosh can modulate estrogen metabolism and inhibit malignant transformation in MCF-10A cells by altering enzyme expression in the chemical carcinogenesis pathway (Fig. 1); this hypothesis was borne out for hops extracts in the present study.
Materials and Methods

Chemicals and reagents

All the chemicals and reagents were obtained from Sigma (St. Louis, MO) or Invitrogen (Carlsbad, CA) unless stated otherwise. All the standard compounds of estrogen metabolites were obtained from Steraloids Inc. (Newport, RI). Antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and cell signaling technology (Boston, MA). Estrone-2,4,16,16-d4, 4-hydroxyestrone-1,2,16,16-d4, and 2-methoxyestrone-1,4,16,16-d4 were obtained from CDN isotope (Pointe-Claire, Quebec) and used as internal standards in estrogen metabolism experiments.

Plant materials and phenolic compounds

Authentic C. racemosa (L.) Nutt. (syn. Actaea racemosa L., black cohosh) rhizomes/roots (BC #192) were acquired through our industrial partner, Naturex, formerly PureWorld Botanicals (South Hackensack, NJ) and were botanically verified and characterized by the UIC/NIH Center for Botanical Dietary Supplements (30). The 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 CO2 extraction of pelletized strobiles of Humulus lupulus cv. Nugget), which was obtained from Hopsteiner (Mainburg [Germany]/New York; Xantho Extract HHE02). 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 and 0.084% 8-PN. XN was isolated and purified (>99.5% purity both by qHNMR and LC-MS) as described previously (26). 8-PN was synthesized and purified (95.0% purity by qHNMR) using the modified literature procedure as previously reported (21).

Cell lines and cell culture conditions

MCF-10A cells were obtained from American Type Culture 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 (15). 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. MCF-10A cells were authenticated using single tandem repeat analysis (STR).

**Analysis of estrogen metabolites in MCF-10A cells**

MCF-10A cells were seeded in 6 well plates at a density of 2000 cells/well. Cells were incubated with E2 (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. Since, hops showed a significant inhibition of estrogen metabolism at 5 µg/mL in MCF-10A cells, a dose response was performed. Different concentrations of hops (1, 2, 5, 7.5, 10 µg/mL) were tested in the presence of E2 (1 µM). The effect of the hops compounds XH and 8-PN on estrogen metabolism were also studied. The dose dependent effect of XH (0.1, 0.5, 1, 2.5, 5 µM) and 8-PN (0.5, 1, 2.5, 5 µM) was tested in the presence of E2 (1 µM).

Treatments were renewed every 3 days. Cell media was collected (5 mL/well) after 3 days of treatment and stored at -20 °C. At the end of the treatment, cell media was collected and pooled with the 3rd day cell media (10 mL/sample total volume). Ascorbic acid (2 mM) and 5 nM of each internal standard (E1-d4, 4-OHE1-d4, and 2-MeOE1-d4) were added into each sample. The derivatization method of Xu et al. was used with minor modification (31) for the sample preparation and analysis. Briefly, the samples were lyophilized to approximately 2 mL aqueous solution and the estrogen metabolites were extracted twice with dichloromethane (4 mL). 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 (10 µL) were analyzed by LC-MS/MS as described below.
**LC-MS/MS method**

All of 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. Estrogen metabolites were quantified using Analyst software (Applied Biosystems, Forster City, CA). Peak areas of 2 and 4-MeOE1 were normalized against 2-MeOE1-d4 internal standard and represented as relative peak area. 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 relative peak area ratio in the graphs.
Cytotoxicity

Cytotoxicity assays were performed in parallel to all the metabolism experiments. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was performed to measure cell viability as described previously (32).

Inhibition of human recombinant CYP450 1B1

Human recombinant CYP450 1B1 isozymes with CYP450 reductase were purchased from Sigma. 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 addition of 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 x 2 mL) and derivatized with dansyl chloride and analyzed by LC-MS/MS as described above for the metabolism experiments. Inhibition of CYP450 1B1 activity was further confirmed using the ethoxyresorufin O-dealkylase assay (supplementary Fig. S1B) as described previously (33) 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.

Immunoblotting

Protein expression of CYP450 1B1 and 1A1 was analyzed using western blot experiments as previously described (34) with minor modifications. Briefly, MCF-10A cells were treated with E₂ (1 µM) in the presence and absence of hops (5 µg/mL) for 1, 3 and 6 days. Cells were harvested and protein lysates were prepared. Protein concentration was determined by
BCA assay. Equal aliquots of total protein samples (30 µg/ lane) were electrophoresed to separate proteins. β-Actin level was measured as a gel loading and transferring control. Anti-CYP450 1B1, anti-CYP450 1A1, anti-COMT and anti-β-actin antibodies were used as primary antibodies in 1:200, 1:1000, 1:1000 and 1:2000 dilution, respectively. 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). 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.

**Anchorage independent growth assay**

To determine the effects of hops and it’s flavonoids on estrogen-induced malignant transformation, anchorage independent growth assay was performed as previously described (15) with minor modifications. Briefly, cells were plated in T-75 flasks at a density of 0.5 x 10^6 cells/flask and treated with hops and its active compounds. Treatment was done with hops (5 µg/mL) or 8-PN (50 nM) in the presence and absence of E2 (1 µM) for 3 weeks, 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. Cells were passaged once a week before confluency was achieved. 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 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 (0.05%) and analyzed using an Olympus inverted microscope (Center Valley, PA). Spherical formation of > 50 cells were taken as a colony. The number of colonies formed in each well were counted and represented as an average of triplicates ± SD.
Statistical analysis

All of the metabolism experiments and western blots were performed in triplicate. All data were expressed as the average ± SD. The statistical analysis of these results consisted of t-test or ANOVA using GraphPad Prism version 5 for Windows.
Results

Analysis of estrogen metabolism in MCF-10A cells

Oxidative estrogen metabolism was assessed by LC-MS/MS and MeOE$_1$ metabolites were measured in MCF-10A cell culture supernatants (Fig. 2, supplementary Fig. S2). Since COMT converts 2-hydroxyestrone and 4-hydroxyestrone into the corresponding 2- and 4-methoxy metabolites (Fig. 1), these served as indices of catechol estrogen formation (35). When the cells were treated with E$_2$ (1 µM) for 6 days and the cell media was analyzed for metabolites, the majority of the metabolites detected were estrone derivatives, while E$_2$ metabolites were much less abundant (supplementary Fig. S2). As a result, the methoxy estrone metabolites (MeOE$_1$) were used as biomarkers for metabolism, since they were stable in cell media compared to the catechol estrogens and could be consistently and reproducibly measured.

Hops inhibits estrogen metabolism in MCF-10A cells while black cohosh had no significant effect

To determine whether hops and black cohosh modulated 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. Since cell viability experiments indicated that 20 µg/mL of hops was toxic to MCF-10A cells (LC$_{50}$ = 11 ± 0.5 µg/mL), hops extract was used at lower concentrations (5 µg/mL) in the metabolism experiments. Black cohosh was not toxic and did not show any significant effect on estrogen metabolism in MCF-10A cells (Fig. 2A). In contrast, co-treatment with hops (5 µg/mL) significantly reduced (p < 0.005) the formation of both 2- and 4-estrone methylethers (Fig. 2B). Hops extract reduced estrogen metabolism in a dose dependent manner in MCF-10A cells (Fig. 3).

Hops modulates estrogen-induced CYP450 enzymes in MCF-10A cells

There are several important CYP450 enzymes involved in the estrogen chemical carcinogenesis pathway (Fig. 1). In breast epithelial cells, the CYP 1 family is mainly involved in
phase I metabolism of E₁ and E₂ (5, 12). 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 (supplementary Fig. S1A, B). In order to determine the effect of hops on E₂ induced P450 enzymes, MCF-10A cells were treated with E₂ (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 (Fig. 4). There was a significant time-dependent induction in CYP450 1B1 and CYP450 1A1 with E₂ treatment with maximum induction at day 6 (Fig. 4). Co-treatment with hops (5 µg/mL) significantly inhibited the induction of both CYP450 1B1 (Fig. 4A) and CYP450 1A1 (Fig. 4B). There was no significant effect on COMT by either E₂ or hops (Fig. 4C).

**Effect of XH and 8-PN on estrogen metabolism**

XH exhibits chemopreventive activity (26), whereas 8-PN shows estrogenic activity (21). 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-MeOE₁ was observed with co-treatment of different concentrations of XH in MCF-10A cells (Fig. 5). In contrast, there was a significant reduction (p < 0.001) in the formation of 2- and 4-MeOE₁ in the presence of nanomolar amounts of 8-PN (Fig. 5).

**Anchorage independent growth inhibition by hops and active compounds.**

The ability to form anchorage independent colonies in soft agar is considered an important hallmark of malignant transformation (14). 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 E₂ treated sample (1 µM) compared to the negative control (0.01% DMSO) (Fig. 6). Co-treatment with hops (5 µg/mL) significantly inhibited (p < 0.005), E₂ induced colony formation in soft agar. Finally, there was also a significant reduction (p < 0.0001) in colony formation with co-treatment with 8-PN (50 nM).
Discussion

In addition to the widely accepted hormonal mechanism of estrogen carcinogenesis, estrogens can be metabolized by CYP450s to form redox active and/or electrophilic o-quinones, which could act as chemical carcinogens by modifying cellular macromolecules (5, 36) (Fig. 1). An imbalance in estrogen metabolism results from increased CYP450-catalyzed o-quinone formation and/or reduced detoxification of these o-quinones, which could affect DNA integrity and lead to transformation of normal cells into a malignant phenotype (17). In the present study, OTC botanical dietary supplements used as alternatives to estrogen replacement therapy, including black cohosh and hops, were analyzed for their ability to modulate oxidative estrogen metabolism in MCF-10A cells. We hypothesized that hops in particular could reduce oxidative estrogen metabolism, transformation of MCF-10A cells, and ultimately prevent estrogen dependent cancers.

Analysis of oxidative estrogen metabolites in biological samples has been reported using a variety of different methods including LC-MS/MS, GC-MS, and immunoassay (31, 37, 38). A LC-MS/MS method was used in the present study in order to accurately measure and quantify 2-MeOE₁ and 4-MeOE₁ in MCF-10A cells (Fig. 2). Since the stability of the catechol estrogens in the cell media was low, the quantity of the methoxy ethers was measured instead, assuming that their production was reflective of catechol estrogen formation (35). Catechol estrogen formation has been measured in MCF-10F cells although pre-exposure to TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) to induce CYP450s 1B1/1A1 was necessary as well as including the COMT inhibitor, Ro-41-0960 in the incubations (39). In the present study, extraction of estrogen metabolites with dichloromethane increased extraction efficiency relative to solid phase extraction, while derivatization with dansyl chloride enhanced the ionization ability in the triple quadruple mass spectrometer leading to higher sensitivity. The LC-MS/MS method used in the present study was sufficiently sensitive to detect 2- and 4-MeOE₁ without any prior induction or inhibition of enzymes in MCF-10A cells.
Estrogen metabolites can be used as biomarkers of cancer initiation and genotoxicity in MCF-10A and 10F cells to provide a platform to better understand the mechanism of estrogen chemical carcinogenesis. These non-tumorigenic human mammary epithelial cell lines have been used as model cellular systems for the analysis of oxidative estrogen metabolism, malignant transformation, DNA modification, and reactive oxygen species (ROS) formation (14, 15, 17, 40, 41). It has been shown that E2-induced oxidative metabolism and malignant transformation can be inhibited by agents with antioxidant activity such as resveratrol (42).

The chemopreventive activities of hops and black cohosh have been investigated and ascribed to induction of detoxification enzymes (26) and antioxidant activity (43), respectively. Black cohosh did not significantly inhibit estrogen metabolism in MCF-10A cells at 20 µg/mL concentration (Fig. 2A). In contrast, hops showed a significant dose-dependent inhibition towards the formation of 2- and 4-MeOE1 (Fig. 2B, 3). Similar modulation of estrogen metabolism has been reported for resveratrol in MCF-10F cells and with dietary berries in animal models (44).

Several potential mechanisms could explain the inhibitory effect of hops on estrogen metabolism. Components of hops could act as direct inhibitors of CYP450 1B1 and 1A1. For example, resveratrol was reported to be an inhibitor of CYP450 1B1 and 1A1, with nanomolar potency (33). 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 (16). Similarly, hops had no effect on CYP450 1B1 estrogen 4-hydroxylase or ethoxyresorufin O-dealkylase activity unless toxic concentrations were used (> 20 µg/mL, supplementary Fig.S1).

Another possible inhibitory mechanism could involve inhibition of E2-induced CYP450 upregulation. 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 (45). Furthermore, CYP450 1A1 expression can also be induced by E2 in HEPA 1C1C7 cells via transcriptional regulation (46). In the present study, a time dependent induction in CYP450 1B1 and 1A1 expression in
MCF-10A cells was observed upon exposure to E₂ (Fig. 4). Since MCF-10A cells do not respond to estrogen via classical ERα genomic pathways, the mechanism of CYP450 induction by E₂ could involve extranuclear ER, ERβ, or aryl hydrocarbon receptor (AhR) since these receptors are expressed in MCF-10 cells (47). In the present study, hops significantly inhibited estrogen-induced CYP450 1B1 and 1A1 expression in MCF-10A cells (Fig. 4).

Since hops showed a significant inhibition in oxidative estrogen metabolism in MCF-10A cells, isolated phenolic components from hops were further studied. XH is the most abundant and bioactive phytoconstituent of hops (48), which has previously been reported to have potential chemopreventive properties via induction of NADPH-dependent quinone oxidoreductase (NQO1) in liver cells (26). However, no significant inhibition of estrogen metabolism by XH was observed in MCF-10A cells (Fig. 5). In contrast, 8-PN which is a potent estrogenic compound isolated from hops (21), showed a significant inhibitory effect (p < 0.0005) even at nanomolar concentrations (Fig. 5). Although 8-PN showed a significant inhibition on estrogen metabolism in MCF-10A cells, it had little inhibitory effect on CYP450 1B1 catalyzed formation of 4-hydroxyestradiol in estradiol metabolism experiments using recombinant CYP450 1B1 enzyme (supplementary Fig. S1A). It has been reported that 8-PN can act via ER-β to modulate gene expression in rat brain cells (49). The mechanism of inhibition of oxidative estrogen metabolism in MCF-10A cells by 8-PN could be through inhibition of E₂ upregulation of CYP450 1B1, which could be mediated through ER-β, extranuclear ER, or AhR.

The ability to form anchorage independent colonies in semi-solid media is considered a characteristics of malignant transformation (14). It has previously been shown that MCF-10A and 10F cells can undergo malignant transformation upon exposure to E₂ (15, 17) and E₂ induced malignant transformation could be inhibited by botanical components such as resveratrol (17, 42). In the present study, it was confirmed that MCF-10A cells were transformed into a malignant phenotype upon exposure to E₂ (Fig. 6). Anchorage independent colony formation in the E₂ treated cells was significantly higher (p < 0.001) than that of the
negative control (0.01% DMSO) (Fig. 6). There was a significant inhibition (p < 0.005) of E2 induced malignant transformation by hops (Fig. 6). 8-PN which is a potent inhibitor of oxidative estrogen metabolism in MCF-10A cells (Fig. 5) also caused a significant reduction (p < 0.0001) in E2-induced malignant transformation when used in nanomolar (50 nM) amounts (Fig. 6). These data suggest that 8-PN could be responsible for the effects of hops extract on E2-induced malignant transformation of MCF-10A cells. The content of 8-PN in hops extracts was 0.09-0.13% (21), which in the doses used in the transformation studies corresponds to approximately 15 - 20 nM 8-PN; quantitatively in accord with the potency observed for inhibition of catechol estrogen formation. These doses are similar to exposure levels expected for women taking hops botanical dietary supplements (50) and the serum levels of prenylflavonoids expected in in vivo studies (21). Furthermore, at these low concentrations, classical antioxidant effects are less likely to be a significant contributor.

In conclusion, hops extract inhibited estrogen oxidative metabolism and estrogen-induced malignant transformation in the MCF-10A model of mammary carcinogenesis. The results are entirely consistent with inhibition mediated by the botanical component and estrogen agonist, 8-PN via potent blockade of estrogen-induced CYP450 upregulation. Further work is needed to distinguish the site of 8-PN interaction, which may be a non-classical ER or AhR; however, these results support the further investigation of hops in humans as a dietary supplement with potential cancer chemopreventive activity in breast epithelial cells.
Acknowledgments

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References


Fig. 1. Scheme of potential mechanisms by which hops can modulate estrogen metabolism in breast epithelial cells. Hops inhibits E₂ induced upregulation of CYP450 1A1 and 1B1 resulting reduced formation of catechol estrogens leading to less quinone production; hence, DNA damage and malignant transformation is decreased. Since catechol O-methyltransferase (COMT) converts 2-hydroxyestrone and 4-hydroxyestrone into the corresponding 2- and 4-methoxy metabolites, these served as indices of catechol estrogen formation.
Fig. 2. Effect of black cohosh and hops on oxidative estrogen metabolism in MCF-10A cells. Positive ion-electrospray selective reaction monitoring (SRM) chromatograms of 2-MeOE\textsubscript{1} and 4-MeOE\textsubscript{1}. (A) Black cohosh had no significant effect on 2-MeOE\textsubscript{1} and 4-MeOE\textsubscript{1} formation while (B) hops significantly inhibited the formation of 2-MeOE\textsubscript{1} and 4-MeOE\textsubscript{1}. MCF-10A cells were treated with and without black cohosh (20 µg/mL) or hops (5 µg/mL) in the presence of E\textsubscript{2} (1 µM) for 6 days. Cell media was collected and the metabolites were extracted in dichloromethane followed by the derivatization using dansyl chloride. Samples were analyzed by LC-MS/MS in multiple reaction monitoring (MRM) mode as described in Materials and Methods. Overlaid SRM chromatograms represent the formation of 2-MeOE\textsubscript{1} and 4-MeOE\textsubscript{1} in E\textsubscript{2} treated (line) and E\textsubscript{2} and (A) black cohosh (dashed line), or (B) hops (dotted line) treated samples.
**Fig. 3.** Dose dependent effect of hops on the formation of 2-MeOE₁ and 4-MeOE₁. MCF-10A cells were treated with different concentrations of hops in the presence of E₂ (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 ± SD of three experiments performed independently in duplicate.
A

![Graph showing relative protein expression over days with CYP450 1B1 and β-Actin expressions below.](image)

- CYP450 1B1
- β-Actin

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- E2
- Hops

- Hops

p < 0.05
B

Relative protein expression

Days
1 3 6

CYP450 1A1

β-Actin

E₂

Hops

p < 0.05
**Fig. 4.** Hops inhibits E₂ induced (A) CYP450 1B1 and (B) 1A1 expression. (C) COMT expression is not significantly affected by either E₂ or hops. Cells were collected at different time points (1, 3, 6 days) after the treatment and protein was extracted and analyzed using immunoblotting. Anti CYP450 1B1, anti CYP450 1A1 and anti COMT antibodies were used in 1:200, 1:1000 and 1:1000 dilutions, respectively. These blots are representatives of three experiments done independently. The intensity of the bands was normalized to β-actin as outlined in Material and Methods and represented as relative protein expression. Each lane contains 30 µg of total protein as determined by BCA assay.
Relative peak area ratio

[Chemical structures of 8-PN and XH]
Fig.5. Effect of XH and 8-PN on estrogen metabolism in MCF-10A cells. 2-MeOE₁ (open circles) and 4-MeOE₁ (closed circles) formation was plotted against different concentrations of XH and 8-PN. MCF-10A cells were treated with E₂ (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 (p< 0.0005) of both 2-MeOE₁ and 4-MeOE₁ formation in the presence of nanomolar concentrations of 8-PN. Each value represents an average ± SD of three experiments performed independently in duplicate.
**Fig. 6.** Effect of hops and 8-PN 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) or 8-PN (50 nM). 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) and 8-PN (p<0.0001) 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.
Hops (Humulus lupulus) Inhibits Oxidative Estrogen metabolism and Estrogen-Induced Malignant Transformation in Human Mammary Epithelial Cells (MCF-10A)


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