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

L.P. Madhubhani P. Hemachandra, R. Esala P. Chandrasena, Shao-Nong Chen, Matthew Main, David C. Lankin, Robert A. Scism, Birgit M. Dietz, Guido F. Pauli, Gregory R.J. Thatcher, and Judy L. Bolton

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–mediated hormonal pathway; and the chemical carcinogenesis pathway involving formation of estrogen quinones that damage DNA and proteins, hence initiating and promoting carcinogenesis. Because, 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. Liquid chromatography/tandem mass spectrometry 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; 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 nmol/L). These data suggest that hops extracts possess cancer chemopreventive activity through attenuation of estrogen metabolism mediated by 8-PN. *Cancer Prev Res;* 1–9. ©2011 AACR.

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; refs. 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; ref. 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 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-hydroxyestradiol (2-OHE2) and 4-hydroxyestradiol (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 nontumorigenic, 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 shown 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 over-the-counter (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).

Materials and Methods

Chemicals and reagents
All the chemicals and reagents were obtained from Sigma or Invitrogen unless stated otherwise. All the standard compounds of estrogen metabolites were obtained from Steraloids Inc. Antibodies were obtained from Santa Cruz Biotechnology and Cell Signaling Technology. 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 Isotopes Inc. 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 Pure World Botanicals 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). The kieselguhr was removed by methanol filtration. Quantitative liquid chromatography/mass spectrometric (LC/MS) analysis using authentic reference compounds as calibrants revealed that this hops extract contained 5.4% XH and 0.084% 8-prenylnaringenin (8-PN). XN was isolated and purified (>99.5% purity both by quantitative HNMR and LC/MS) as described previously (26). 8-PN was synthesized and purified (95.0% purity by quantitative HNMR) 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 and maintained in Dulbecco’s Modified Eagle’s Medium and F12 medium (DMEM/Ham’s F-12) supplemented with 1% penicillin-streptomycin, 5% FBS, cholera toxin (0.1 μg/mL), epidermal growth factor (20 ng/mL), hydrocortisone (0.5 μg/mL), insulin (10 μg/mL), and 5% CO₂ at 37°C as described previously (15). Estrogen-free medium for MCF-10A cells were prepared supplementing charcoal-dextran-treated FBS to phenol red-free DMEM/Ham’s F-12, whereas other components remain the same. MCF-10A cells were authenticated by single tandem repeat analysis.

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

MCF-10A cells were seeded in 6-well plates at a density of 20,000 cells per well. Cells were incubated with E₂ (1 μmol/L) in the presence or absence of hops (5 μg/mL) and black cohosh (20 μg/mL) for 6 days. Because 20 μg/mL of hops showed toxicity in MCF-10A cells, lower concentrations of hops (5 μg/mL) were used for all the experiments. Because, hops showed a significant inhibition of estrogen metabolism at 5 μg/mL in MCF-10A cells, a dose response was carried out. Different concentrations of hops (1, 2.5, 5, 7.5 and 10 μg/mL) were tested in the presence of E₂ (1 μmol/L). The effect of the hops compounds XH and 8-PN on estrogen metabolism were also studied. The dose-dependent effect of XH (0.1–2.5 μmol/L) and 8-PN (0.5–2.5 μmol/L) was tested in the presence of E₂ (1 μmol/L).

Treatments were renewed every 3 days. Cell media were collected (5 mL/well) after 3 days of treatment and stored at −20°C. At the end of the treatment, cell media were collected and pooled with the third-day cell media (10 mL/sample total volume). Ascorbic acid (2 mmol/L) and 50 mmol/L potassium phosphate buffer (pH 7.4), and either hops (20 μg/mL) or 8-PN (1 μmol/L) was preincubated at 37°C for 5 minutes. The reaction was initiated by adding NADPH (1 mmol/L) and dansyl chloride (1 mg/mL in acetone). The reaction mixture was incubated at 60°C for 10 minutes to complete the derivatization. Samples (10 μL) were analyzed by liquid chromatography/tandem mass spectrometry (LC/MS-MS) as described later.

**LC/MS-MS method**

All of the metabolism experiments were carried out by positive ion electrospray tandem mass spectrometric methodology on an API 3000 (Applied Biosystem) triple quadruple mass spectrometer equipped with Agilent 1200 HPLC (Agilent Technologies). Liquid chromatography was carried out on a 150 mm × 3 mm i.d. column packed with 3.5-μm particles, XBridge C-18 column (Waters). The mobile phase, operating at the flow rate of 300 μL per minute 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-minute run were set at 80% solvent B. The chromatographic gradient was held at the initial conditions for 5 minutes followed by a linear gradient of B from 80% to 95% over 20 minutes and held at 95% solvent B for 5 minutes. The mass spectrometer parameters were optimized as follows: the ion-spray 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 was 370 V, and the declustering potential was 81 V. The collision energy for 2-methoxyestriene (2-MeOE₁), 4-methoxyestriene (4-MeOE₁), and 2-MeOE₁-d₄ was 59 V, whereas for 2-hydroxyestriene (2-OHE₁), 4-hydroxyestriene (4-OHE₁) and 4-OHE₁-d₄, it was 51 V. Collision energy for 4-MeOE₁ and 2-MeOE₁-d₄, respectively. Estrogen metabolites were quantified with Analyst software (Applied Biosystems). Peak areas of 2-MeOE₁ and 4-MeOE₁ were normalized against 2-MeOE₁-d₄ internal standard and represented as relative peak area. 2-MeOE₁ 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 conducted in parallel to all the metabolism experiments. MTT assay was conducted 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/L), E₂ (5 μmol/L), potassium phosphate buffer (50 mmol/L, pH = 7.4), and either hops (20 μg/mL) or 8-PN (1 μmol/L) was preincubated at 37°C for 5 minutes. The reaction was initiated by adding NADPH (1 mmol/L).
into each reaction mixture and incubated for 1 hour 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 minutes). Ascorbic acid (2 mmol/L) was added into each sample before 4-OHE1-β-glucuronidase (5 mmol/L) 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 earlier (33) with minor modifications. Briefly, recombinant CYP450 1B1 was incubated with E2 (1 μmol/L) and NADPH (1 mmol/L) in potassium phosphate buffer (50 mmol/L, 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 minutes and determined as percentage CYP450 1B1 activity calculated from the resorufin standard curve.

**Immunoblotting**

Protein expression of CYP450 1B1 and CYP450 1A1 was analyzed by Western blot experiments as previously described (34) with minor modifications. Briefly, MCF-10A cells were treated with E2 (1 μmol/L) 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 bicinchoninic acid (BCA) assay. Equal aliquots of total protein samples (30 μg per 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, 1:1,000, 1:1,000, and 1:2,000 dilution, respectively. Antibodies were diluted in blocking solution (5% nonfat milk in TBS with 0.1% Tween 20). Blots were incubated with primary antibody overnight at 4°C and with secondary antibody for 1 hour at room temperature. Blots were visualized with chemiluminescence substrate (Thermo Scientific). Imaging and analysis was done with FluoroChem software (Cell Biosciences). 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 its flavonoids on estrogen-induced malignant transformation, anchorage-independent growth assay was conducted as previously described (15) with minor modifications. Briefly, cells were plated in T-75 flasks at a density of 0.5 × 10⁶ cells per flask and treated with hops and its active compounds. Treatment was done with hops (5 μg/mL) or 8-PN (50 nmol/L) in the presence and absence of E2 (1 μmol/L) for 3 weeks, twice a week. Dimethyl sulfoxide (DMSO; 0.01%) was used as the vehicle control in the experiments, whereas E2 (1 μmol/L) 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 × 10⁴ cells per well in 12-well plates precoated with 0.6% agar base medium. Estrogen-free media were added as the feeding media on top of the soft agar layer. Cells were maintained in soft agar for 3 weeks, and media were refreshed every 3 days. After 3 weeks, colonies were stained with crystal violet (0.05%) and analyzed by an Olympus inverted microscope. Spherical formation of more than 50 cells was 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 blot analysis were carried out in triplicate. All data were expressed as the average ± SD. The statistical analysis of these results consisted of t test or ANOVA by 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 MeOE1 metabolites were measured in MCF-10A cell culture supernatants (Fig. 2, Supplementary Fig. S2). Because COMT converts 2-OHE1 and 4-OHE1 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 E2 (1 μmol/L) for 6 days, and the cell media were analyzed for metabolites, the majority of the metabolites detected were estrone derivatives, whereas E1 metabolites were much less abundant (Supplementary Fig. S2). As a result, the methoxy estrone metabolites (MeOE) were used as biomarkers for metabolism, as they were stable in cell media compared with the catechol estrogens and could be consistently and reproducibly measured.

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

To determine whether hops and black cohosh modulated estrogen metabolism, MCF-10A cells were treated with E2 (1 μmol/L) in the presence or absence of either hops (5 μg/mL) or black cohosh extracts (20 μg/mL) for 6 days. Because cell viability experiments indicated that 20 μg/mL of hops were toxic to MCF-10A cells [lethal concentration 50 (LC50) = 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, cotreatment 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 these data showed that toxic concentrations were necessary before any significant inhibition of estradiol metabolism or ethoxyresorufin O-dealkylation activity was observed (Supplementary Fig. S1A and S1B). To determine the effect of hops on E2-induced P450 enzymes, MCF-10A cells were treated with E2 (1 μmol/L) 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). Cotreatment 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 xanthohumol and 8-prenylnaringenin on estrogen metabolism**

XH exhibits chemopreventive activity (26), whereas 8-PN shows estrogenic activity (21). Because pure standards of these compounds were available, they were tested for their effect on estrogen metabolism. No significant reduction in the formation of 2-MeOE and 4-MeOE was observed with cotreatment 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-MeOE and 4-MeOE 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 μmol/L) compared with the negative control (0.01% DMSO; Fig. 6). Cotreatment 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 cotreatment with 8-PN (50 nmol/L).

**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, gas chromatography–mass spectroscopy, and immunoassay (31, 37, 38). A LC/MS-MS method was used in the present study to accurately measure and quantify 2-MeOE1 and 4-MeOE1 in MCF-10A cells (Fig. 2). Because 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 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 and derivatization with dansyl chloride enhanced the ionization ability in the triple quadruple mass spectrometer leading to the treatment, and protein was extracted and analyzed by immunoblotting. Anti-CYP450 1B1, anti-CYP450 1A1, and anti-COMT antibodies were used in 1:200, 1:1,000, and 1:1,000 dilutions, respectively. These blots are representatives of 3 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 bicinchoninic acid assay.

Figure 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 μmol/L) in the presence and absence of different concentrations of either XH or 8-PN for 6 days. Cell media were analyzed for estrogen metabolites by 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 3 experiments carried out independently in duplicate.

Figure 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, and 6 d) after the treatment, and protein was extracted and analyzed by immunoblotting. Anti-CYP450 1B1, anti-CYP450 1A1, and anti-COMT antibodies were used in 1:200, 1:1,000, and 1:1,000 dilutions, respectively. These blots are representatives of 3 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 bicinchoninic acid assay.
to higher sensitivity. The LC/MS-MS method used in the present study was sufficiently sensitive to detect 2-MeOE1 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 MCF-10F cells to provide a platform to better understand the mechanism of estrogen chemical carcinogenesis. These nontumorogenic 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 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-MeOE1 and 4-MeOE1 (Fig. 2B; Fig. 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 CYP450 1A1. For example, resveratrol and its derivatives were reported to be inhibitors of CYP450 1B1 and CYP450 1A1, with nanomolar potency (33). However, similar studies in CYP450 1B1 and CYP450 1A1 suprasomes, 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 ethoxysresorufin 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 estrogen responsive element 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 CYP450 1A1 expression in MCF-10A cells was observed upon exposure to E2 (Fig. 4). Because MCF-10A cells do not respond to estrogen via classical ERα genomic pathways, the mechanism of CYP450 induction by E2 could involve extranuclear ER, ER-β, or aryl hydrocarbon receptor (AhR), as these receptors are expressed in MCF-10 cells (47). In the present study, hops significantly inhibited estrogen-induced CYP450 1B1 and CYP450 1A1 expression in MCF-10A cells (Fig. 4).

Because 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-OH-E2 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 semisolid 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 < 0.003) of E2-induced malignant transformation upon exposure to E2 + hops (Fig. 6).
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 \(E_2\)-induced malignant transformation when used in nanomolar (50 nmol/L) amounts (Fig. 6). These data suggest that 8-PN could be responsible for the effects of hops extract on \(E_2\)-induced malignant transformation of MCF-10A cells. The content of 8-PN in hops extracts was 0.084%, which in the doses used in the transformation studies corresponds to approximately 15 to 20 nmol/L 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 nonclassical ER, ER-\(\beta\) 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.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**

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