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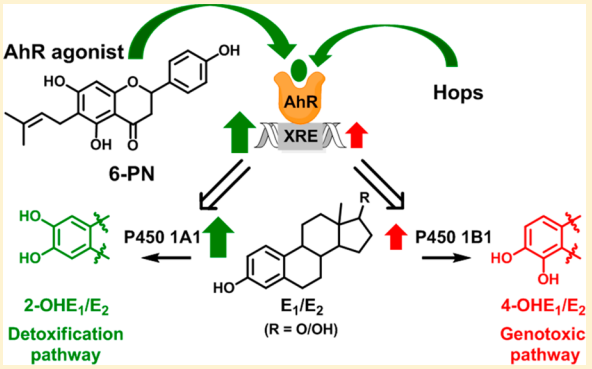
Hop (*Humulus lupulus* L.) Extract and 6-Prenylnaringenin Induce P450 1A1 Catalyzed Estrogen 2-Hydroxylation

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

UIC/NIH Center for Botanical Dietary Supplements Research, Department of Medicinal Chemistry and Pharmacognosy, University of Illinois at Chicago, 833 South Wood Street, Chicago, Illinois 60612-7231, United States

Supporting Information

ABSTRACT: *Humulus lupulus* L. (hops) is a popular botanical dietary supplement used by women as a sleep aid and for postmenopausal symptom relief. In addition to its efficacy for menopausal symptoms, hops can also modulate the chemical estrogen carcinogenesis pathway and potentially protect women from breast cancer. In the present study, an enriched hop extract and the key bioactive compounds [6-prenylnarigenin (6-PN), 8-prenylnarigenin (8-PN), isoxanthohumol (IX), and xanthohumol (XH)] were tested for their effects on estrogen metabolism in breast cells (MCF-10A and MCF-7). The methoxyestrones (2-/4-MeOE$_1$) were analyzed as biomarkers for the nontoxic P450 1A1 catalyzed 2-hydroxylation and the genotoxic P450 1B1 catalyzed 4-hydroxylation pathways, respectively. The results indicated that the hop extract and 6-PN preferentially induced the 2-hydroxylation pathway in both cell lines. 8-PN only showed slight up-regulation of metabolism in MCF-7 cells, whereas IX and XH did not have significant effects in either cell line. To further explore the influence of hops and its bioactive marker compounds on P450 1A1/1B1, mRNA expression and ethoxyresorufin *O*-dealkylase (EROD) activity were measured. The results correlated with the metabolism data and showed that hop extract and 6-PN preferentially enhanced P450 1A1 mRNA expression and increased P450 1A1/1B1 activity. The aryl hydrocarbon receptor (AhR) activation by the isolated compounds was tested using xenobiotic response element (XRE) luciferase construct transfected cells. 6-PN was found to be an AhR agonist that significantly induced XRE activation and inhibited 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) induced XRE activity. 6-PN mediated induction of EROD activity was also inhibited by the AhR antagonist CH223191. These data show that the hop extract and 6-PN preferentially enhance the nontoxic estrogen 2-hydroxylation pathway through AhR mediated up-regulation of P450 1A1, which further emphasizes the importance of standardization of botanical extracts to multiple chemical markers for both safety and desired bioactivity.



INTRODUCTION

Hormone therapy (HT) has been the standard treatment option for postmenopausal symptom relief for decades.[1] However, HT has been linked with increased breast cancer risk in a number of clinical trials including the Women's Health Initiative (WHI).[2−4] As a natural and perceived safe alternative to HT for postmenopausal system relief, botanical dietary supplements have been increasingly popular.[5,6] However, rigorous interdisciplinary studies on the efficacy, potential toxicity, and health benefits of these botanicals continue to be in high demand.

Prolonged exposure to estrogens including HT increases breast cancer risk.[7−9] The two major mechanisms of carcinogenesis are estrogen signaling (hormonal pathway) and metabolism of estrogens to reactive quinones (chemical pathway, Figure 1). Recently, a number of cohort studies analyzing the risk correlation between estrogen levels, estrogen metabolites, and breast cancer risk in postmenopausal women were conducted.[10−12] The results indicated that higher estrogen levels were associated with increased risk of postmenopausal breast cancer, while enhanced estrogen 2-hydroxylation suggested a lower risk for breast cancer. Various *in vitro* studies have supported this finding that estrogen 2-hydroxylation represents a detoxification pathway, whereas 4-hydroxylation is correlated with malignant transformation.[13−15]

P450 1A1/1B1 are the major extra-hepatic P450 1 enzymes that metabolize estrogens into 2- or 4-hydroxylated forms, respectively (Figure 1).[16] The expression is mainly controlled by the upstream aryl hydrocarbon receptor (AhR), which translocates into the nucleus upon activation and binds to the xenobiotic response element (XRE), initiating targeted gene transcription.[17] In breast tissues, these P450s are directly correlated with the local estrogen metabolism.[7−9] The 2- and 4-

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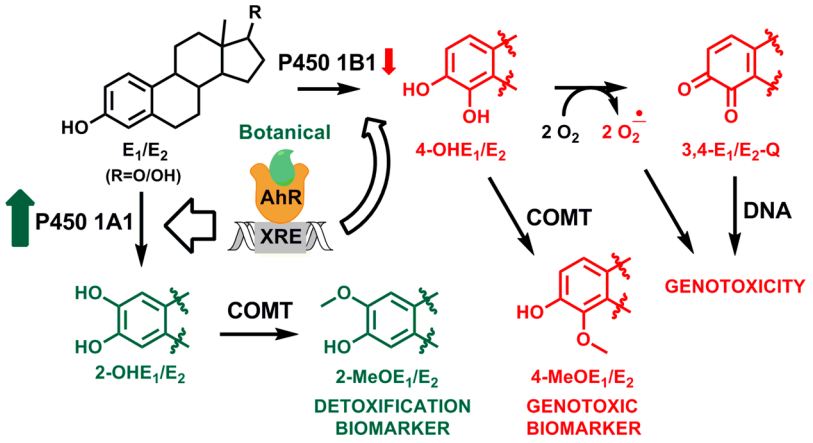

Figure 1. Hypothesis: effect of botanicals on estrogen chemical carcinogenesis. P450 1A1 catalyzes the formation of 2-OHE_1/E_2 (detoxification biomarker), which are clinically shown to be correlated with reduced breast cancer risk. P450 1B1 catalyzes the formation of 4-OHE_1/E_2 (genotoxic biomarker), which is oxidized to the genotoxic estrogen-3,4-quinone (3,4-E_1/E_2-Q) and forms ROS through redox cycling. The reactive quinone and ROS contribute to estrogen carcinogenesis. Chemopreventive botanicals are hypothesized to increase 2-hydroxylation and decrease 4-hydroxylation metabolism, as shown with green and red arrows.

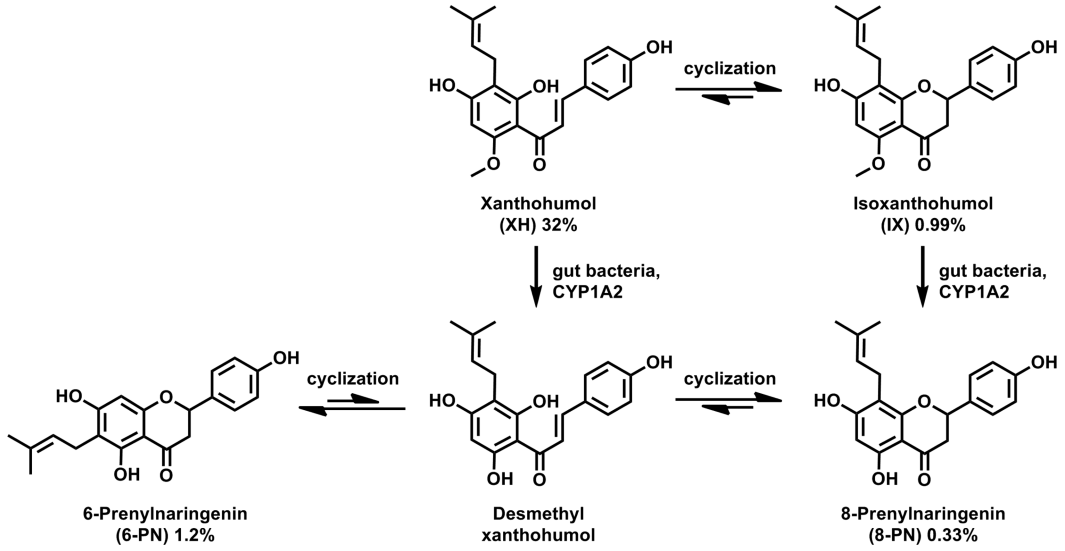

Figure 2. Key bioactive compounds in hops.

hydroxylated estrogen catechols can be further metabolized by catechol-O-methyl transferase (COMT) to the more stable 2- and 4-methoxy ether metabolites, which can be used as biomarkers for 2- and 4-hydroxylation pathways.[18,19]

Hops (strobiles of *Humulus lupulus* L., Cannabaceae) have been traditionally used as a sleep aid and, more recently, by women for postmenopausal symptom relief.[20,21] Many biological activities of hops have been connected to a series of bioactive prenylated flavanones and chalcones such as 6-prenylnarigenin (6-PN), 8-prenylnarigenin (8-PN), isoxanthohumol (IX), and xanthohumol (XH) (Figure 2).[22,23] The predominant prenylated chalcone, XH, has been shown to be an effective chemopreventive agent, inducing the detoxification enzyme NAD(P)H:quinone oxidoreductase 1 (NQO1) in both *in vitro* and *in vivo* studies.[23−25] XH is metabolized to IX, desmethyl xanthohumol, 8-PN, and 6-PN as shown in Figure 2.[26,27] 8-PN has been reported to be one of the most potent estrogen receptor alpha (ERα) phytoestrogens known to date, which is likely responsible for menopausal symptom relief.[22,28]

Previously, we showed that a hop extract was able to reduce the potentially genotoxic estrogen 4-hydroxylation pathway and decrease estradiol (E_2) induced colony formation in human nontumorigenic breast epithelial MCF-10A cells.[18] The UIC/NIH Center for Botanical Dietary Supplements Research has further enriched a spent hop extract with respect to its estrogenic (8-PN) and chemopreventive (XH) compounds (Figure 2).[29,30] The new standardized extract contained much higher levels of the marker compounds than the previous extract. The purpose of this study was to test the effect of this standardized hop extract, which has been used in human clinical trials,[31] on estrogen metabolism. Since a relatively low response was observed in MCF-10A cells, we also included the well characterized breast cancer MCF-7 cell line to confirm the bioactivities of the standardized hop extract as well as the effects of the four major prenylated marker compounds. The effects of this new hop extract and compounds on estrogen oxidative metabolism in the two breast cell lines were studied. Their effects on P450 1A1/1B1 mRNA expression and activity in MCF-10A and MCF-7 cells and inhibition of recombinant

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P450 1A1/1B1 activity were also measured. Finally, XRE activation was analyzed in both liver HepG2 cells and MCF-7 cells to confirm the mechanism of action. The results suggest that hops can selectively enhance P450 1A1 catalyzed estrogen 2-hydroxylation and potentially reduce breast cancer risk.

MATERIALS AND METHODS

Chemicals and Reagents. All chemicals and reagents were purchased from Sigma (St. Louis, MO) or Invitrogen (Carlsbad, CA) unless otherwise stated. S enantiomers of 6-PN and 8-PN were purchased from Sigma (St. Louis, MO). The ethanol extract of botanically authentic spent strobiles of *Humulus lupulus* was obtained from Hopsteiner (Mainburg, Germany, and New York, NY) and standardized to the prenylated polyphenol marker compounds 6-PN, 8-PN, IX, and XH as previously described (Figure 2).[29,30] Briefly, standardization involved characterization by LC-UV, LC-MS/MS, and quantitative ^{1}H NMR (qHNMR). The same extract has been used in a Phase I clinical trial in postmenopausal women.[31] The concentrations of the four marker compounds in this extract were 1.2% 6-PN, 0.33% 8-PN, 0.99% IX, and 32% XH.

Cell Lines and Culture Conditions. MCF-10A and MCF-7 cells were obtained from American Type Culture Collection (Manassas, VA, USA). MCF-10A cells were cultured in 1:1 Dulbecco's modified Eagle medium and Ham's F12 nutrient mixture (DMEM/F12) with 15 mM HEPES and L-glutamine (Invitrogen), supplemented with 20 ng/mL epidermal growth factor, 100 ng/mL cholera toxin, 0.5 μg/mL hydrocortisone, 10 μg/mL insulin, 5% horse serum, and 1% penicillin−streptomycin at 37 °C with 5% CO_2 as described previously.[32] MCF-7 cells were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum, 1% glutamax, 1% AB/AM, 1% nonessential amino acids, and 3 μg/mL insulin. All experiments were done with cells under 15 passages in phenol-red free media supplemented with charcoal stripped serum and the same other ingredients.

Preparation of Estrogen Metabolite Samples. Estradiol and all of the standard compounds of estrogen metabolites were obtained from Steraloids Inc. (Newport, RI). 4-Methoxyestrone-1,4,16,16-d4 was obtained from CDN isotope (Pointe-Claire, Quebec) and used as internal standard in the estrogen metabolites analysis. 2-Methoxyestrone and 4-Methoxyestrone were measured as indicators of the level of estrogen 2-hydroxylation and the 4-hydroxylation pathway as previously described with modifications.[32,33] Briefly, cells were estrogen starved for 24 h before being seeded into 6-well plates. Cells were incubated with E_2 (1 μM) in the presence and absence of hop extract (0.1−2.5 μg/mL) and 6-PN, 8-PN, IX, and XH (0.1−6 μM) for 2 days. Cell media were then collected and spiked with 0.4 nM internal and 2 mM ascorbic acid. The media were then extracted with 2 × 4 mL of dichloromethane. The organic layers were then combined and dried under a gentle flow of nitrogen. Derivatization was performed with 100 μL of 0.1 M $NaHCO_3$ buffer (pH 9.5) and 100 μL of dansyl chloride in acetone (1 mg/mL) at 60 °C in a water bath with agitation for 10 min. Samples were then cooled on ice, and 50 μL of sample was analyzed by LC-MS/MS as described below.

Analysis of Estrogen Metabolites by LC-MS/MS. After derivation, all samples were analyzed by a positive ion electrospray tandem mass spectrometric method as previously described.[32] Briefly, LC-MS/MS was performed by using an Agilent 1200 series nano flow LC system (Agilent Technologies, Aanta Clara, CA) coupled to an AB SCIEX Triple Quad 5500 System (AB SCIEX, Framingham, MA). The liquid chromatography separation was carried out with a 100 mm × 3 mm i.d. Waters BEH C-18 column packed with 1.7 μm particles (Waters, Milford, MA) and maintained at 40 °C. Multiple reactions monitoring transitions were selected as follows: 534.4−171.2 for the detection of dansylated $MeOE_1$ and 538.4−171.2 for dansylated $MeOE_1$-d4. Quantitation was performed using the Analyst software (Applied Biosystems, Forster City, CA), and data were normalized to the E_2 control treatment in each independent experiment.

Quantification of P450 1A1/1B1 mRNA Expression via qPCR. MCF-10A and MCF-7 cells at a density of 2.5 × 10^5 cells/mL were plated in 6-well plates and treated with DMSO, hops, 6-PN, 8-PN, IX, or XH for 24 h. RNA extraction, reverse transcription, and PCR were performed according to manufacturers' protocols as previously described.[32] The total RNA was extracted according to Qiagen's (Valencia, CA) RNeasy kit instructions. RNA was reverse transcribed according to Invitrogen's SuperScript III First-Strand Synthesis System for RT-PCR. The resulting cDNA (2 μL) was used for real-time PCR quantification using Applied Biosystems' (Carlsbad, CA) StepOnePlus Real-Time PCR System. Taqman gene expression master mix and P450 1B1 primer with FAM/MGB probe from Applied Biosystems were added to a 96-well reaction plate with cDNA to perform real-time quantitative PCR (one cycle of 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min). Data were analyzed with the comparative C_T (ΔΔCT) method to determine fold difference in reference to the HPRT1 endogenous control.

Ethoxyresorufin-*O*-deethylase Activity Assay. EROD assay measuring P450 1 enzyme activity was conducted both in cells and with recombinant P450 1A1 and 1B1 enzymes as previously described.[34,35] Briefly, 1 × 10^5 cells/well were plated and treated with hop extract or compounds for 2 days, cells were washed with PBS and incubated with 2.5 μM 7-ethoxyresorufin and 1.5 mM salicylamide in PBS at 37 °C. Fluorescence was measured every minute with excitation at 530 nm and emission at 590 nm for 25 min with a BioTek (Winooski, VT) Synergy H4 Hybrid Multi-Mode Microplate Reader. For enzyme inhibition experiments, recombinant P450 1A1 and 1B1 protein with reductase were purchased from BD Biosciences (Woburn, MA). P450 1A1 (0.15 pmole) or 1B1 (0.8 pmole) was preincubated with test compounds or vehicle for 5 min at 37 °C in 200 μL of 50 mM potassium phosphate buffer (pH 7.4) with 1 mM NADPH. Negative controls were done in parallel without NADPH. Fluorescence signals were not affected by these compounds in the inhibition experiments. The reaction was initiated by adding 7-ethoxyresorufin solution in potassium phosphate buffer to a final concentration of 2.5 μM. IC_{50} and apparent K_i (K_i') values were calculated. Fluorescence was measured every minute after 5 s of mixing for 25 min at 37 °C. Fluorescence with 7-ethoxyresorufin as substrate was linear for more than 15 min, and the reaction rate was determined from the slope of the linear regression curves plotted with data points measured in the first 15 min.

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

Statistics. The data were expressed as the mean ± SEM of at least three independent experiments. Significance was determined using one-way ANOVA with Dunnett's post-test, comparing treatment groups to the control (*$p < 0.05$).

RESULTS

Hop Extract and 6-PN Preferentially Induced Estrogen 2-Hydroxylation Metabolism in MCF-10A and MCF-7 Cells. Previously, we showed that a hop extract slightly decreased the estrogen 4-hydroxylation pathway and had no effect on the 2-hydroxylation pathway in MCF-10A cells in a six day experiment.[18] In the current study, two day metabolism studies with the new clinical hop extract described in the Material and Methods showed that this hop extract stimulated

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estrogen 2-hydroxyaltion (Figure 3A). One problem with the MCF-10A cell line is its relatively low activities of P450 1A1/1B1.[36] In order to confirm the qualitative effects of hop extract and the bioactive marker compounds, additional experiments were done with the well characterized MCF-7 cells, which are known to be much more sensitive for P450 1A1/1B1 inductions.[36] The data showed much higher overall induction of metabolism (10−20-fold), and 2-MeOE$_1$ formation was preferred similar to the MCF-10A data (Figure 3B). Although the preferential induction was observed in both cell lines, the differences in MCF-10A cells were more prominent than in MCF-7 cells with both hops and 6-PN treatment. 6-PN was the most potent compound tested, inducing 2-MeOE$_1$ 50-fold compared to 40-fold for 4-MeOE$_1$ in MCF-7 cells and 3.5-fold for 2-MeOE$_1$ induction versus 2-fold for 4-MeOE$_1$ in MCF-10A cells (Figure 3C and D). In contrast to the MCF-10A cell experiments, 8-PN showed moderate induction of estrogen metabolism in MCF-7 cells, which could be due to the higher AhR mediated P450 1A1/1B1 induction in MCF-7 cells. XH and IX did not have significant effects in either cell line. Overall, the results in MCF-7 cells are comparable with the MCF-10A data and suggested that hops and 6-PN preferentially induce estrogen 2-hydroxylation metabolism in breast cells.

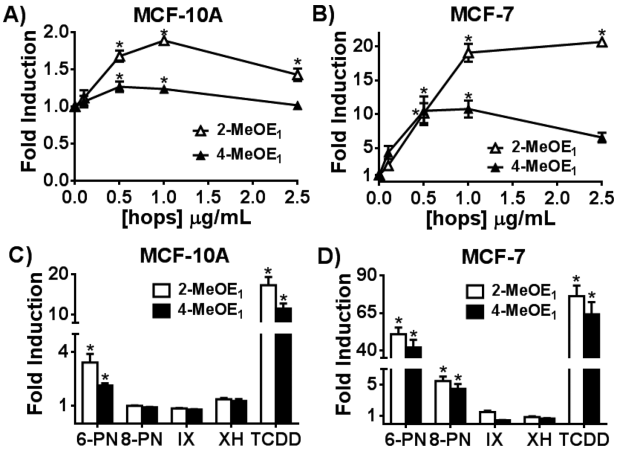

Figure 3. Hop extract and 6-PN preferentially induced 2-hydroxylation metabolism in breast MCF-10A and MCF-7 cells. (A) MCF-10A cells and (B) MCF-7 cells were treated with E_2 (1 μM) and the hop extract for 2 days, and media were collected and analyzed for 2-MeOE$_1$ and 4-MeOE$_1$ metabolite level by LC-MS/MS. (C) MCF-10A cells and (D) MCF-7 cells were treated with E_2 (1 μM) and 6-PN, 8-PN, IX, XH (1 μM), and TCDD (10 nM) for 2 days, and media were analyzed for 2-MeOE$_1$ and 4-MeOE$_1$ metabolites. Results were normalized to fold induction against estradiol treated cells. Data were plotted as the means ± SEM of three independent experiments and analyzed by one-way ANOVA with Dunnett's multiple comparison post-test to compare treatment groups with the control group, *$p < 0.05$.

Hop Extract and 6-PN Preferentially Induced P450 1A1 mRNA Expression in MCF-10A and MCF-7 Cells. P450 1A1/1B1 mRNA levels were analyzed 24 h after treatment with hops and the bioactive compounds. In MCF-10A cells, hop extract significantly induced P450 1A1 mRNA expression to 7-fold, with no significant effect on 1B1 (Figure 4A). In MCF-7 cells, the induction levels were significantly higher with 90- and 35-fold induction of P450 1A1 and 1B1 mRNA expression (Figure 4B). Regarding the hop compounds, the only compound that significantly increased P450 1A1 and 1B1 was 6-PN, with an increase to 16- and 2-fold, respectively,

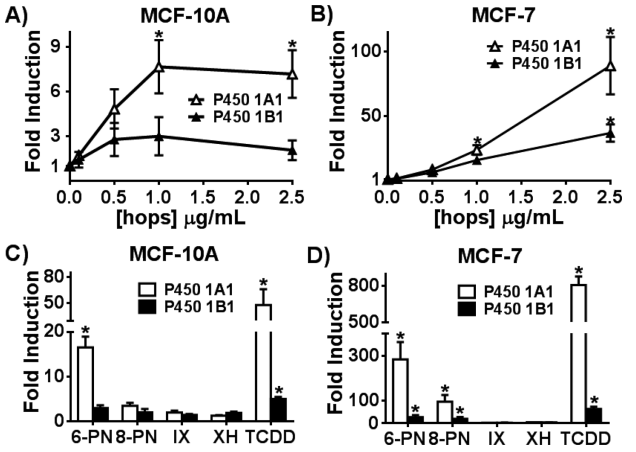

Figure 4. Hop extract and 6-PN preferentially induced P450 1A1 mRNA expression in MCF-10A and MCF-7 cells. (A) MCF-10A cells and (B) MCF-7 cells were treated with hop extract, and P450 1A1 and 1B1 mRNA expression was analyzed after 24 h via qPCR. (C) MCF-10A and (D) MCF-7 cells were treated with 6-PN, 8-PN, IX, XH (1 μM), and TCDD (10 nM) for 24 h, and P450 1A1/1B1 mRNA expression was analyzed via qPCR. Results were plotted as the means ± SEM of three independent experiments and analyzed by one-way ANOVA with Dunnett's multiple comparison post-test to compare treatment groups to the DMSO control, *$p < 0.05$.

in MCF-10A cells (Figure 4C). In MCF-7 cells, qPCR analysis also showed that 6-PN preferentially increased P450 1A1 mRNA levels to around 290-fold compared to the 25-fold induction of P450 1B1 (Figure 4D). 8-PN significantly induced P450 1A1 and 1B1 in MCF-7 cells (Figure 4D), yet not in MCF-10A cells (Figure 4C), and this induction in MCF-7 cells (90-fold and 20-fold, respectively) was less than that of 6-PN. IX and XH did not show significant effects in either cell line. Although these prenylated polyphenols share some common structural moieties, their bioactivities show remarkable differences. These data correlate with the results from estrogen oxidative metabolism, indicating that hops and 6-PN preferentially increased P450 1A1 mRNA levels in breast cells.

Hop Extract and 6-PN Induced P450 1A1/1B1 Activity in MCF-10A and MCF-7 Cells. P450 1A1/1B1 activity was measured using the EROD assay in both cell lines after 2 days of treatment with hop extract and bioactive compounds. In MCF-10A cells, in the presence of the hop extract, a significant dose−responsive induction was observed to a maximum of 0.04 pmol/min/well resorufin formed (Figure 5A). In MCF-7 cells, the hop extract gave significantly higher EROD activity compared to MCF-10A cells to a maximum of 0.25 pmol/min/well resorufin (Figure 5B). With the hop compounds in MCF-10A cells, only 6-PN increased P450 1A1/1B1 activity dose-dependently to 0.05 pmol/min/well resorufin (Figure 5C). XH moderately induced the P450 1 activity even though estrogen metabolism and P450 1A1/1B1 gene expression were not affected; 8-PN and IX did not show significant effects. In MCF-7 cells, significant induction of P450 1A1/1B1 activity was observed with 6-PN and 8-PN (3 μM) to 0.8 and 0.2 pmol/min/well resorufin, while IX and XH did not have significant effects (Figure 5D). Overall, the results from the EROD activity assay were consistent with the results from estrogen metabolism and P450 1A1/1B1 mRNA analysis. Qualitatively, the data from these two cell lines were also comparable and indicated that hops and 6-PN strongly induced P450 1A1/1B1 activity in breast cells.

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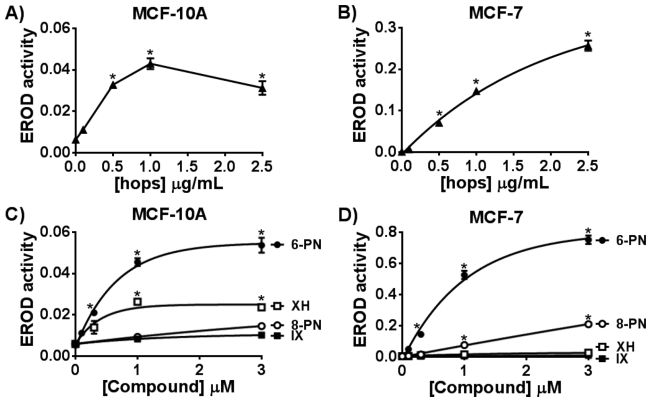

Figure 5. Hop extract and 6-PN increased P450 1A1/1B1 activity in MCF-10A and MCF-7 cells. P450 1A1/1B1 activity was analyzed in MCF-10A cells after 2-day treatment of (A) hop extract and (C) different doses of 6-PN, 8-PN, IX, and XH with the EROD assay. P450 1A1/1B1 activity was analyzed in MCF-7 cells after 2-day treatment of (B) hop extract and (D) different doses of 6-PN, 8-PN, IX, and XH with the EROD assay. Results were represented as pmol/min/well resorufin formed and plotted as the means ± SEM of three independent experiments and analyzed by one-way ANOVA with Dunnett's multiple comparison post-test to compare treatment groups to DMSO control, $*p < 0.05$.

6-PN Increased XRE Activation and Acted as an AhR Agonist. Human hepatoma HepG2 cells and MCF-7 cells transfected with a XRE-luciferase construct were used to measure the effect of bioactive compounds on AhR activation. The cells were incubated with the compounds for 24 h after transient transfection of the luciferase construct. Of the four polyphenols, 6-PN and 8-PN significantly and dose-dependently increased XRE-luciferase activity to around 6.5- and 10-fold of the control at 10 μM, while XH and IX did not have significant effects (Figure 6A). In the presence of the AhR agonist TCDD (10 nM), a dose−responsive decrease in XRE luciferase activity was observed with 6-PN and 8-PN cotreatment (Figure 6B), which further suggested the interactions with AhR. In MCF-7 cells, the XRE response was lower than that in HepG2 cells (Figure 6C). 6-PN (5 μM) significantly induced the activity, while 8-PN (5 μM) had no effect, which correlated with the P450 1A1/1B1 activity and gene expression analysis. The results indicated some cell selectivity of 8-PN and also suggested 6-PN to be activating AhR in both breast and liver cell lines. Finally, upon cotreatment of AhR antagonist CH223191 and 6-PN (1 μM) in MCF-7 cells, the EROD activity was inhibited dose−responsively (Figure 6D), which further confirmed 6-PN to be an AhR agonist. In summary, these results suggested that 6-PN acted as an AhR agonist in both breast and liver cells.

Hop Compounds Inhibit Recombinant P450 1A1/1B1 Activity but Had Only Moderate P450 1A1/1B1 Inhibition in Cells. Various flavonoids have been previously reported to inhibit P450 1A1/1B1 activities.[37−39] To study the inhibitory effects of hop compounds on P450 1A1/1B1, the EROD assay with recombinant enzymes was conducted. All four compounds acted as P450 1A1/1B1 inhibitors with IC_{50} values in the low micromolar range without selectivity for either P450 1A1 or 1B1 (Table 1). However, the inhibitory activity of these compounds in cell culture experiments was considerably less than that with recombinant enzymes (Figures S2 and S3). These data indicated the metabolism results were mainly the result of induction of P450 1A1/1B1 enzyme levels and that little direct inhibition of P450s should be observed at clinical concentrations of hop supplements.

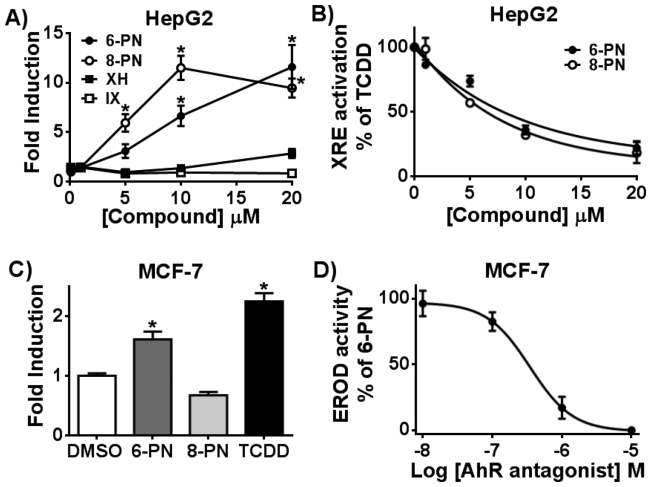

Figure 6. 6-PN induced XRE-luciferase activity and acted as a partial AhR agonist. HepG2 cells were incubated with (A) hop compounds alone and (B) 6-PN and 8-PN in the presence of TCDD (10 nM) for 24 h before analysis of XRE-luciferase reporter activity. (C) MCF-7 cells were incubated with 6-PN, 8-PN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

Table 1. Inhibition of Recombinant P450 1A1/1B1 Enzymes with Hop Compounds[a]

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

[a]The values are expressed as the means ± SD from three independent dose−responsive curves using recombinant P450 1A1 and P450 1B1 enzymes.

DISCUSSION

Estrogen exposure has long been linked with postmenopausal breast cancer risk, especially since the WHI report in 2002.[2,8,9] Estrogen carcinogenesis includes the hormonal mechanism involving classical ER binding and estrogen signaling which promotes cell growth and the chemical mechanism where estrogens are converted to reactive quinones which modify DNA leading to genotoxicity (Figure 1).[7−9] The estrogen 4-hydroxylation pathway is considered the genotoxic pathway by forming the electrophilic/redox active estrogen-3,4-quinone and reactive oxygen species (ROS) which causes DNA damage.[40,41] Contrary to estrogen 4-hydroxylation, estrogen 2-hydroxylation is a nongenotoxic pathway, and the metabolite, 2-Methoxyestradiol, has been shown to have antiproliferative/anticancer activity.[13] Several recent clinical trials analyzing serum estrogen metabolite levels and the risk of postmenopausal breast cancer further support the 2-hydroxylation pathway as a marker for chemoprevention; however, the data are inconclusive on the relationship between the estrogen 4-hydroxylation and breast cancer risk.[10−12,15]

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P450 1A1 and 1B1 are the major enzymes in breast tissues that are responsible for the local estrogen 2- and 4-hydroxylation metabolism, respectively.[42,43] These two enzymes share about 40% homology and are generally co-upregulated upon AhR activation.[44] AhR is also responsible for the expression of several other Phase I and Phase II enzymes, which are associated with the detoxification of environmental carcinogens as well as the potential activation of pro-carcinogens.[45] P450 1B1 expression levels in tumors, as well as carcinogen-induced P450 1B1 levels in cancer cells, are higher than in normal tissues and cells.[46−48]

MCF-10A cells have been frequently used as a model to study estrogen chemical carcinogenesis due to the absence of ER and they present a nontumorigenic phenotype.[8,32,49−51] However, the AhR mediated P450 1A1/1B1 induction in MCF-10A cells is relatively low as reported previously[36] and confirmed in the present study. Data also suggest that the Phase I and Phase II enzyme expression is variable in MCF-10A cells depending on the confluence level in cell culture.[52] In addition, spontaneous expression of ER might occur in MCF-10A cells after a certain number of passages.[53] These potential problems and the variability observed in the current experiments prompted additional studies in the more robust MCF-7 cells.

MCF-7 cells have been used in estrogen metabolism studies by numerous investigators.[51,54,55] Spink et al. showed that induction of P450 1A1/1B1 enzymes vary among different tumorigenic and nontumorigenic cell lines. They determined that MCF-7 cells had higher levels of TCDD-induced P450 1A1/1B1 mRNA and almost 8-fold higher metabolic rates for TCDD-induced 4-MeOE$_2$ formation compared to those of MCF-10A cells.[36] Similarly, our results showed more than 15-fold increase in P450 1A1/1B1 gene induction with TCDD treatment in MCF-7 over MCF-10A cells (Figure 4C and D) and about 10-fold higher metabolite formation with hop extract treatment in MCF-7 compared to that of MCF-10A cells (Figure 3A and B). Since MCF-7 cells mirrored MCF-10A cells in the response to botanical treatment with higher induction levels, they serve as a better model to screen botanicals and compounds for the modulation of estrogen metabolism. In addition, the upstream P450 1A1/1B1 mRNA induction trend among compounds (6-PN ≫ 8-PN and no effect with IX and XH) corresponds with the metabolism and activity results in MCF-7 cells. However, due to the presence of ER there is potential crosstalk between AhR and ERα signaling pathways, and AhR agonists have been reported to increase proteasomal degradation of ER.[56,57] The interesting relationship between ERα, AhR, and botanical modulation of estrogen metabolism will be the subject of future studies.

Botanicals have been previously shown to affect AhR activation, and the major compounds responsible were polyphenols.[58,59] For example, resveratrol has been shown to induce XRE activation to about 6-fold at 10 μM in MCF-10A cells.[50] In this study, HepG2 cells were used to compare the AhR activation by hop compounds because the XRE-luciferase reporter activity in HepG2 cells were much higher compared to that in MCF-7 cells (Figure S7). Comparatively, in this study XRE-luciferase activity was increased to 6.5-fold by 6-PN (10 μM) in HepG2 cells (Figure 6A). 8-PN displayed some cell selectivity with about 10-fold XRE activation at 10 μM in HepG2 cells but showed no effect in MCF-7 cells. In addition, hop compounds also showed differential effects on P450 1A1/1B1 regulation. Preferential induction of P450 1A1 over P450 1B1 has been documented in the literature. Quercetin (10 μM) and berberine (>5 μM) preferentially induced P450 1A1 over 1B1 in MCF-10F and MCF-7 cells, respectively.[60,61] In contrast, benzo(a)pyrene (1 μM) preferentially increased P450 1B1 in human oral epithelial cells.[62] However, the mechanisms of P450 1A1 and 1B1 preferential induction as well as *in vivo* effects need to be further studied. 6-PN significantly increased P450 1A1/1B1 activity and mRNA expression. Induction of XRE activity and inhibition of TCDD induced XRE activity further supported 6-PN to be an AhR agonist (Figure 6). Induction of P450 1 enzymes by natural flavonoids and flavonoid rich botanicals have been reported previously.[58] Naringenin, a flavanone with the same scaffold as the hop flavanones, does not induce P450 1A1/1B1,[59,63] while the effect of flavanones with a prenylated side chain has not been studied.[64] Results from this study indicated that prenylated naringenin derivatives exhibit a unique activity compared to that of the parent naringenin. In addition, the significant P450 1A1/1B1 induction by 6-PN compared to 8-PN may suggest that the positioning of the prenyl group is important for AhR activation.

The effect of hop compounds on TCDD-induced XRE-luciferase reporter activity was also measured.[65,66] The results indicated that 6-PN and 8-PN had comparable effects in inhibiting TCDD induced XRE-luciferase activity at micromolar levels (Figure 6B). Resveratrol, as well as the scaffold parent compound naringenin, also demonstrated significant inhibitory effects on TCDD induced EROD activity above 10 μM in MCF-10A cells.[50,63] Similar studies looking at estrogen metabolism using a MCF-10F cell model observed decreased formation of 4-MeOE$_1$/E$_2$ and DNA adducts after cotreatment with resveratrol (25 μM) and TCDD (10 nM).[67] In contrast, we previously showed that licochalcone A (10 μM), a B-ring (C-5′) prenylated chalcone from licorice (*Glycyrrhiza inflata*), was an AhR antagonist and shut down estrogen oxidative metabolism in MCF-10A cells.[32]

Various botanical flavonoids have been identified as inhibitors of P450 enzymes.[37−39] It has also been shown that flavonoids generally exhibit more potent P450 1B1 inhibition over 1A1.[68] Several ubiquitous, unsubstituted flavones and flavonols, such as quercetin, kaempferol, and apigenin, have been reported to be P450 1B1 inhibitors with IC$_{50}$ values below 50 nM, while inhibiting P450 1A1 activity less potently.[37,39,68] However, flavanones, like narigenin, are generally weak P450 1 inhibitors with IC$_{50}$ values in the micromolar range, which might indicate the importance of the C-ring 2−3 double bond in P450 1 enzyme inhibition.[37,69] Several hydroxychalcones have been tested for the inhibition of P450 1A1 and 1B1 and showed IC$_{50}$ values in the low micromolar range.[70] Our results as well as previous studies from Henderson et al. showed that the prenylflavanones from hops are more potent inhibitors compared to the parent flavanone, naringenin.[35] The hop compounds had similar inhibitory activities toward P450 1B1, with IC$_{50}$ values around 0.5 μM; 6-PN showed around 3-fold lower inhibitory activity to P450 1A1 (IC$_{50}$ 0.6 μM) than 1B1 (IC$_{50}$ 0.2 μM) (Table 1). When compared to resveratrol, which inhibited P450 1A1 and 1B1 with IC$_{50}$ values around 2 and 25 μM, respectively, these hop compounds are more potent P450 1 inhibitors.[50] The hop compounds have also been shown to inhibit P450 2C8, 2C9, and 19 with IC$_{50}$ values in the low micromolar range.[71,72] However, in contrast to the experiments with purified P450s, the hop compounds showed little to no inhibition in cells (Figure S3), likely due to extensive

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metabolism.[26,27] These data suggest that the effect on estrogen oxidative metabolism modulation was mainly contributed by the AhR agonist activity of 6-PN. The results from this study suggests that hop extracts should be standardized not only to 8-PN for estrogenic effects and to XH for chemopreventive properties but also to 6-PN for its potential modulation of estrogen metabolism. However, further studies are needed to test and confirm the activities *in vivo*.[31,73]

In conclusion, results from this study provided novel *in vitro* evidence that hops and its compound 6-PN preferentially induced the nontoxic estrogen 2-hydroxylation pathway in two different breast cell lines, which indicated a potentially protective role of hops to help reduce the risk of breast cancer through estrogen metabolism modulation. As hop dietary supplements are taken widely by women for postmenopausal symptom relief, it is important to expand our knowledge about the bioactivity and safety of 6-PN and related hop compounds. The clinical trial data have indicated long half-lives of these hop compounds.[31] Since the pharmacokinetic properties of the hop compounds would significantly influence their effect *in vivo*, the preferential 2-hydroxylation induction as well as modulation of P450 1A1/1B1 enzymes would need to be tested with *in vivo* models that will be studied in the future. The present data also confirm the importance of performing botanical standardization to several bioactive phyto-constituents simultaneously. Accordingly, for hop extracts, suitable target markers are 8-PN (estrogenic), XH (chemopreventive), and, as shown in the present study, 6-PN (AhR agonist) as modulators of estrogen metabolism. Assessing the levels of these compounds in standardized hop extracts will be beneficial for the health effects and enhance the safety of women consuming these herbal preparations. Collectively, the present findings provide additional rationales for a meaningful chemical and biological standardization of safe and effective hop botanical supplements.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemrestox.6b00112.

EROD activity in MCF-7 cells treated with hop compounds in the exact amount as in the tested hop extract, the dose−response curves of P450 1A1 and 1B1 recombinant enzyme inhibition, P450 1 enzyme inhibition with hop compounds in cells, COMT expression upon treatment of hop compounds, dose−response curves of 2-MeOE$_1$ and 4-MeOE$_1$ formation with hop compound treatment in MCF-10A cells, EROD activity with TCDD treatment in MCF-10A and MCF-7 cells, XRE-luciferase activity with TCDD treatment in MCF-7 and HepG2 cells, time-dependent EROD induction with TCDD treatment in MCF-7 cells, and calculated apparent K_i values from recombinant P450 1A1/1B1 inhibition (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Department of Medicinal Chemistry and Pharmacognosy, University of Illinois at Chicago, 833 S. Wood Street, M/C 781, Chicago, IL 60612-7231. Phone: 312-996-5280. Fax: 312-996-7107. E-mail: Judy.Bolton@uic.edu.

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■ ABBREVIATIONS

AhR, aryl hydrocarbon receptor; COMT, catechol *O*-methyl transferase; E_1, estrone; E_2, estradiol; ER, estrogen receptor; EROD, ethoxyresorufin *O*-deethylation; HT, hormone therapy; IX, isoxanthohumol; 2-MeOE$_1$, 2-methoxyestrone; 4-MeOE$_1$, 4-methoxyestrone; NQO1, NAD(P)H:quinone oxidoreductase 1; P450 1A1, cytochrome P450 enzyme 1A1; P450 1B1, cytochrome P450 enzyme 1B1; 6-PN, 6-prenylnaringenin; 8-PN, 8-prenylnaringenin; ROS, reactive oxygen species; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; WHI, women's health initiative; XH, xanthohumol; XRE, xenobiotic response element

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