

The Multiple Biological Targets of Hops and Bioactive Compounds

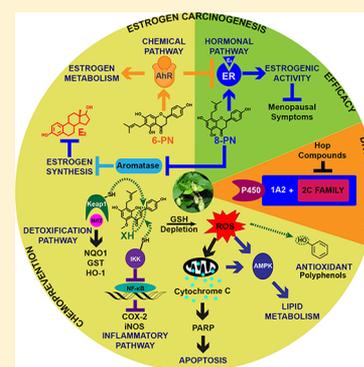
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ABSTRACT: Botanical dietary supplements for women's health are increasingly popular. Older women tend to take botanical supplements such as hops as natural alternatives to traditional hormone therapy to relieve menopausal symptoms. Especially extracts from spent hops, the plant material remaining after beer brewing, are enriched in bioactive prenylated flavonoids that correlate with the health benefits of the plant. The chalcone xanthohumol (XH) is the major prenylated flavonoid in spent hops. Other less abundant but important bioactive prenylated flavonoids are isoxanthohumol (IX), 8-prenylnaringenin (8-PN), and 6-prenylnaringenin (6-PN). Pharmacokinetic studies revealed that these flavonoids are conjugated rapidly with glucuronic acid. XH also undergoes phase I metabolism *in vivo* to form IX, 8-PN, and 6-PN. Several hop constituents are responsible for distinct effects linked to multiple biological targets, including hormonal, metabolic, inflammatory, and epigenetic pathways. 8-PN is one of the most potent phytoestrogens and is responsible for hops' estrogenic activities. Hops also inhibit aromatase activity, which is linked to 8-PN. The weak electrophile, XH, can activate the Keap1-Nrf2 pathway and turn on the synthesis of detoxification enzymes such as NAD(P)H-quinone oxidoreductase 1 and glutathione S-transferase. XH also alkylates IKK and NF- κ B, resulting in anti-inflammatory activity. Antiobesity activities have been described for XH and XH-rich hop extracts likely through activation of AMP-activated protein kinase signaling pathways. Hop extracts modulate the estrogen chemical carcinogenesis pathway by enhancing P450 1A1 detoxification. The mechanism appears to involve activation of the aryl hydrocarbon receptor (AhR) by the AhR agonist, 6-PN, leading to degradation of the estrogen receptor. Finally, prenylated phenols from hops are known inhibitors of P450 1A1/2; P450 1B1; and P450 2C8, 2C9, and 2C19. Understanding the biological targets of hop dietary supplements and their phytoconstituents will ultimately lead to standardized botanical products with higher efficacy, safety, and chemopreventive properties.



1. INTRODUCTION

Hops, the strobili of *Humulus lupulus L.* (Cannabaceae), are added to beer during its preparation and are largely responsible for the taste.^{1–3} Hops are added in the form of dried plant material or as lipophilic extracts. Relatively hydrophilic hop extracts are also used in phytotherapy as one of the ingredients of herbal sedative/sleeping pills and as a natural alternative to traditional hormone replacement formulations to relieve menopausal symptoms.^{4–6} Hop extracts are generally obtained from the female hop cones, and the extracts used for the production of dietary supplements are particularly rich in prenylated phenols including chalcones and flavanones (“flavonoids;” Figure 1).^{1,7} This review will focus on preparations from spent hop cones, representing the material remaining after extraction of the much more lipophilic hop bitter acids, prenylated phloroglucinols, and the essential oils that are important for beer brewing.⁸ Xanthohumol (XH) is the most highly concentrated phenolic hop constituent. However, as all of the hop flavonoids are biochemically interconvertible (Figure 1),

varying ratios of metabolites are observed depending on the extract preparation. XH has various anticarcinogenic properties, shown *in vitro* at key stages of the carcinogenic process including initiation, promotion, and progression phases.^{9,10} In addition to XH, many biological activities of spent hops have been connected to the other prenylated flavanones and chalcones (Figure 1) including 8-prenylnaringenin (8-PN), 6-prenylnaringenin (6-PN), and isoxanthohumol (IX).^{1,11}

This review evaluates the biological targets of spent hops and connects the designated major bioactive constituent to the respective bioactivity. After presenting the unique phytochemistry of spent hops, this review will summarize the current knowledge about pharmacokinetic data of spent hop extracts and their individual flavonoids/chalcones. Subsequently, the text covers the diverse biological targets starting with the action of 8-PN on the estrogen receptor (ER) and on aromatase, followed

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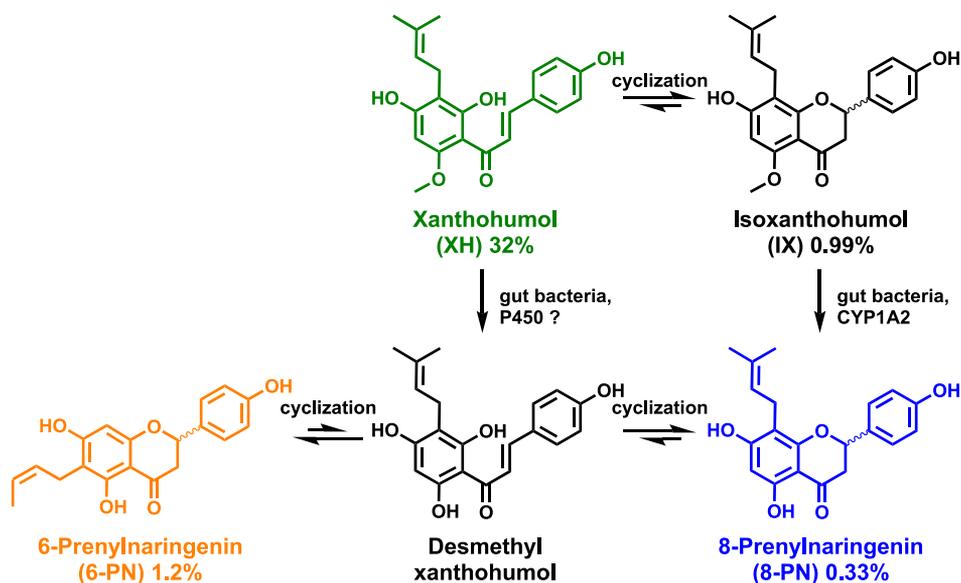


Figure 1. Main bioactive compounds in the standardized spent hop extract. The concentrations of the compounds in the hop extract were determined as described in ref 8. P450 1A2 and the human gut bacterium *Eubacterium limosum* have been described to metabolize IX to 8-PN.^{25,26} While P450 mediated *O*-demethylation of XH to DMX is theoretically possible, it has not been analyzed in detail and might not be clinically relevant.²⁰

by 6-PN's agonistic properties at the aryl hydrocarbon receptor (AhR), and XH's chemopreventive as well as antiglycemic activities. Evidence for potential herb–drug interactions of hops are analyzed by looking at CYP P450 inhibition studies with hop extracts and prenylated phenols from hops. The final section provides a summary of methodology for optimizing spent hop extracts to contain enriched amounts of beneficial bioactive compounds and/or reduced levels of constituents with less desirable effects, collectively leading to hop extracts with enhanced efficacy and safety. It should be emphasized that hop extracts represent a metabolomic mixture that contains thousands of compounds, all of which are likely to have biological activities on their own or in combination with other compounds. This polypharmacological activity of hops is responsible for its rich biological history.³

2.a. Phytochemistry of Hops. The term “hops” is diffuse as it may generally refer to one or more entire plant, or just to the strobili (female inflorescences) of *Humulus lupulus* L., or to any of a multitude of proprietary preparations used in brewing. Hop strobili are generally known in commerce as “hop cones,” whereas the proper pharmacognostic term is *Strobili lupuli*. Being distinct from the composition of the rest of the plant, the phytochemical constituents of *Strobili lupuli* have been studied extensively.¹² Both chemical knowledge and product development have been mainly driven by the brewing industry, as hops have been considered an essential ingredient in beer for >500 years.¹³ The large economic footprint and inherent competition in the industry led to extensive research and rigorous optimization of the sensory properties of the hop bitter acids (prenylated phloroglucinol derivatives), from all aspects of hop production from agronomic factors such as breeding and farming practices through harvesting, drying, storage, extraction, formulation, and dosing. Initial development of hop extracts for brewing employed traditional solvent extraction. By the 1980s, hop extracts had become one of the driving forces in the development of the supercritical fluid extraction (SFE) industry. SFEs are regarded as a more stable, efficient, and consistent manner to impart hop bitterness into beer and

are employed by most large breweries worldwide. Notably, employing hop SFEs in brewing produces large amounts of spent hops as a byproduct—which is well suited as a starting material for the biomedical research described herein.⁸

2.b. Dried Entire Hops vs Spent Hops. Anecdotal observations of “side effects” associated with whole hops, such as estrogenic effects, have sparked further interest in the bioactivity and chemical diversity of the plant.¹³ Early investigators categorized hop constituents broadly into the volatile essential oil, “soft resins,” and “hard resins.”¹⁴ The essential oil and soft resins are responsible for most hop flavor in beer. The soft resins are primarily composed of the bitter acids along with waxes, fats, and entrained volatile terpenoids. The role of the hard resins in brewing and beer flavor has long been controversial. However, there is consensus that most of their constituents, and notably the key phytoestrogenic compound, 8-PN, as well as its precursors, remain in the plant material after CO₂ SFE and, therefore, are not part of the prevalent beer brewing processes that rely on hop SFEs as ingredients. Accordingly, spent hops contain the entirety of the phytoestrogenic potential of this plant, which has primarily been attributed to 8-PN (Figure 1),¹⁵ a compound that remains the most potent phytoestrogen known to date. Other notable phytoconstituents of spent hops include the “protoestrogen” desmethylxanthohumol (DMX), which isomerizes to form 8-PN and/or the isomeric, 6-PN, IX, and its precursor XH (Figure 1). Collectively, these considerations make spent hops an ideal starting material for a botanical dietary supplement as it can be produced from the byproduct of a food manufacturing process, and because it has favorable health effects related to bioactive compounds that are much less important for or even unrelated to brewing beer.⁸

2.c. Phytochemistry of Spent Hops. Among the many pharmacologically active components identified to date in spent hops, the prenylated chalcone, XH, is one of the most abundant and most widely studied phytoconstituents.^{5,7} This is due to both its abundance in hops and its attraction as a natural chemopreventive agent. An intramolecular Michael

addition leads to isomerization and establishes both close biogenetical and chemical relationships between the hop chalcones (XH, DMX) and their corresponding flavanones (IX, 8-PN, 6-PN).¹⁶ To date, all evidence indicates that chalcones are the end of the actual biosynthetic pathways, whereas the flavanones arise through chemical isomerization of their natural chalcone precursors (Figure 1).

3. PK PROPERTIES OF HOP BIOACTIVE COMPOUNDS

Following oral administration, hop prenylated phenols are absorbed through the intestinal epithelium at a slow to moderate rate. Studies using the Caco-2 human intestinal epithelial model indicate that the flavonoid 8-PN crosses the intestinal epithelium at a moderate rate via passive diffusion,¹⁷ while the chalcone XH accumulates in intestinal epithelial cells and enters the general circulation more slowly.¹⁸ The Caco-2 cell monolayer model studies also indicated that glucuronidation as well as sulfation of these compounds can occur in the intestinal epithelium. Cell based studies using human liver microsomes and primary human hepatocytes have shown that phase II conjugation predominates over phase I metabolism and that glucuronidation is the primary route of conjugation.^{17,19}

In clinical trials using orally administered extracts of spent hops or isolated hop prenylated phenols, conjugated metabolites were abundant in serum and urine, whereas only traces of unconjugated compounds or phase I metabolites (<10% of the administered dose) were observed.^{20,21} Similarly, after oral administration of a hop extract, no phase I metabolites but predominantly glucuronides of hop prenylated phenols and only traces of the aglycones were measured in human breast tissue.²²

Urinary excretion of glucuronides and sulfates accounted for <2% of the administered dose of hop prenylated phenols in 24 h²¹ and <8% in 48 h.²³ During the first 48 h after oral administration to humans, up to 24% of the administered doses of hop prenylated phenols were recovered in feces,²³ indicating biliary secretion of conjugated metabolites followed by fecal elimination was the primary route of excretion. However, the slow rate of excretion suggested enterohepatic circulation.

The pharmacokinetics of hop bioactive compounds have been studied using isolated, single prenylated phenols as well as with extracts of spent hops containing mixtures.^{20,21,23} Similar pharmacokinetics results have been obtained for studies of mixtures as well as for investigations of individual hop compounds, indicating no evidence of PK interactions between hop constituents. The maximum serum concentrations of bioactive hop compounds were dose dependent and occurred 1 to 7 h after oral administration.^{20,21} Secondary peaks in serum concentration were observed 4 to 5 h later, indicating enterohepatic circulation. Slow absorption and enterohepatic circulation of these bioactive hop compounds resulted in half-lives exceeding 20 h.^{20,21}

Quantitative analysis of multiple bioactive hop compounds during pharmacokinetic studies using a standardized extract of spent hops in women²¹ indicated some cyclization of XH occurred to form IX as well as some demethylation of IX to form 8-PN. Human pharmacokinetics studies with pure XH confirmed *in vivo* formation of IX and trace amounts of the subsequent IX metabolite 8-PN.²⁰ An investigation of the pharmacokinetics of pure IX in rats confirmed *in vivo* formation of 8-PN from IX.²⁴ Detailed metabolism studies have demonstrated that P450s, mainly P4501A2, as well as gut microbiota can metabolize IX to 8-PN (Figure 1).^{25,26} *Eubacterium limosum* is the gut bacterium mainly responsible

for this metabolism. It is present in the colon of many humans.²⁶ As the concentration of *E. limosum* varies strongly between individuals, the final *in vivo* concentration of the phytoestrogen 8-PN may also differ significantly in a diverse population.²⁶ While the metabolism of XH to DMX by P450 or microbiota is theoretically possible (Figure 1), it might not be clinically relevant. DMX would quickly cyclize to both 8-PN and 6-PN.¹⁶ After oral administration of purified XH, no 6-PN has been observed in human subjects, indicating that *O*-demethylation of XH and cyclization to 6-PN is not a major metabolic pathway in humans.²⁰ A recent pharmacokinetic study in healthy women and men administering a single dose of 500 mg of pure 6-PN or 8-PN revealed that 8-PN plasma levels were significantly higher than 6-PN plasma concentrations.²⁷

4. ESTROGEN RECEPTOR (ER) AS A BIOLOGICAL TARGET

Hops contain the potent phytoestrogen 8-PN, which binds tighter to ER α compared to ER β .^{28,29} In an animal model of menopausal hot flashes, 8-PN, similar to estradiol, restored the normal tail skin temperature of ovariectomized animals.³⁰ This suggests that 8-PN may also lead to menopausal symptom relief in women.^{5,31} 8-PN's concentration in crude plant extracts is at least 10 times lower than XH;³² however, *in vivo*, more 8-PN is likely formed from XH (Figure 1). Also, the concentration in hormone sensitive tissues (e.g., breast) could be considerably higher due to active transport mediated by ER α .³³ In several hormone responsive cell-based assays (Figure 2) as well as

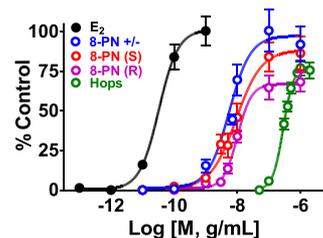


Figure 2. Estrogenicity of 8-PN enantiomers compared with estradiol and the standardized spent hop extract. ER α endometrial carcinoma cells (Ishikawa) were grown in 96 well plate (5×10^4 cells/well) in estrogen-free media for 24 h. Subsequently, they were treated with the spent hop extract and compounds at varied concentrations for 96 h at 37 °C, then washed and lysed. Induced estrogen-dependent alkaline phosphatase activity was evaluated spectrophotometrically at 405 nm by the absorbance of the formed *p*-nitrophenol from the *p*-nitrophenol phosphate substrate (1 mg/mL). Estrogenic activity of respective treatments was assessed by comparison to the estradiol control (1 nM). The results represent the mean \pm SD of at least three independent experiments in triplicate for each sample. The dose response curves were generated using GraphPad Prism.

animal model studies, 8-PN exhibited estrogenic activity.^{28,34–36} Using the procedure of Pisha et al., as shown in Figure 2, the racemic mixture of the *R/S* forms of 8-PN had the highest estrogenic activity.³⁷ 8-PN is in part synthesized from IX *in vivo* (Figure 1) and it has been shown that *R*- and *S*-8-PN can be formed from IX *in vivo*.²⁴ An animal study described that while a standardized extract of spent hops containing 8-PN did not increase uterine weight in a rat model, pure 8-PN did increase uterine weight and the height of luminal epithelial cells.^{34,38} The prenyl group in 8-PN is likely responsible for its high ER α potency and efficacy, since naringenin lacks the 8-prenyl side chain and is much less potent than 8-PN.³⁹ Prenylation

increases the hydrophobicity of 8-PN, which optimizes binding in the hydrophobic pocket of ER α . Prenylation also enhances the PK profile of 8-PN as discussed above. In ovariectomized rat models, a standardized spent hop extract did not show estrogenic effects on the uterus and on the mammary gland.^{40,41} However, weak bone protective effects on bone loss occurred after 8 weeks of treatment with the standardized hop extract (60 mg/kg BW/d) indicating beneficial estrogenic activity.⁴⁰

5. AROMATASE AS A BIOLOGICAL TARGET (FIGURE 3)

Aromatase is the rate-limiting enzyme in the generation of estradiol/estrone in the mammary gland (Figure 3). As a

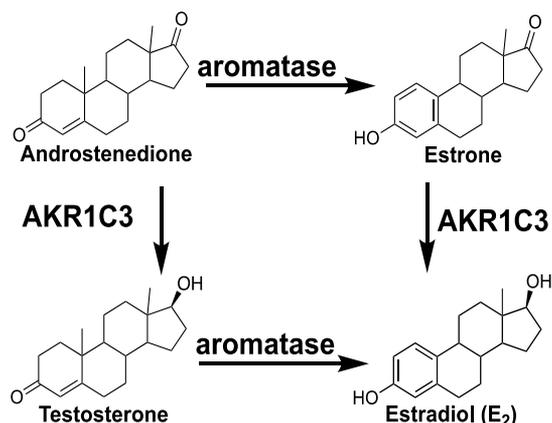


Figure 3. Formation of estrone/estradiol catalyzed by aromatase and aldoketoreductase 1C3 (AKR1C3).

result, inhibiting aromatase with dietary supplements can serve as a chemopreventive mechanism particularly in obese menopausal women.^{42–44} Previous work has shown that the phytoestrogen, 8-PN, is a potent inhibitor of aromatase with an IC₅₀ = 0.08 μ M.^{45,46} This value is in the same range as our data, which gave an approximate IC₅₀ of 0.06 μ M (Figure 4).

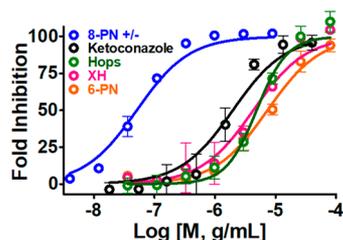


Figure 4. Inhibition of aromatase by hops and hop compounds. Aromatase activity was determined using the CYP19/MFC High-Throughput Inhibitor Screening Kit according to the manufacturer's protocol (Corning, Woburn, MA). The NADPH-cofactor mix was prepared and added to the 96-well plate. The standardized spent hop extract, the hop compounds, and the positive control, ketoconazole, were dissolved in acetonitrile, added to the 96-well plate, and preincubated for 10 min after serial dilutions were performed. The compounds were then incubated with the enzyme/substrate mix (CYP19/7-methoxy-4-trifluoromethyl coumarin) for 30 min. The stop reagent was added thereafter, and the fluorescent signal was read at an excitation of 409 nm and emission of 530 nm. The data represent the average \pm SD of three experiments. The dose response curves were generated using GraphPad Prism.

As shown in Figure 4, the spent hop extract had similar activity to the positive control ketoconazole. XH and 6-PN were also tested, and they were weak aromatase inhibitors. It has been

speculated that the potent inhibitor activity of 8-PN might be due to its structural similarity to both the substrates and products of the aromatase reaction (Figure 3).^{45,47} Finally, different kinds of beer have also been shown to inhibit aromatase in choriocarcinoma derived JAR cells.⁴⁶

6. THE ARYL HYDROCARBON RECEPTOR (AHR) AS A BIOLOGICAL TARGET (FIGURE 5)

Hops may influence not only estrogen synthesis but also estrogen metabolism through the AhR pathway. It has been shown that a standardized spent hop extract²¹ can alter P450 1A1/1B1-mediated estrogen metabolism in malignant (MCF-7) and nontumorigenic mammary epithelial cells (MCF-10A).⁴⁸ The relative levels of the estrogen metabolites, 2-methoxyestrone (2-MeOE₁) and 4-methoxyestrone (4-MeOE₁), generated via the detoxification and genotoxic pathways, respectively, were studied via LC-MS/MS analysis (Figure 5). These metabolites

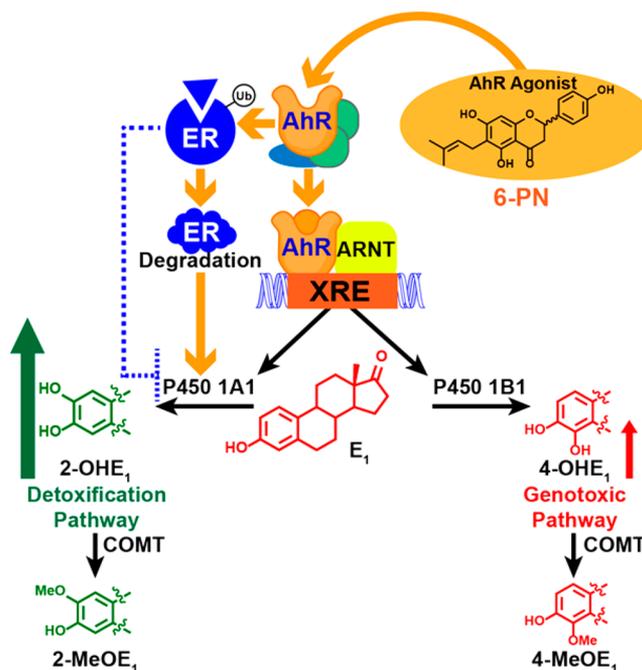


Figure 5. 6-PN, an AhR agonist leading to the induction of P450 1A1. 6-PN increases estrogen metabolism after AhR-mediated upregulation of estrogen metabolizing enzymes, P450 1A1 and P450 1B1. AhR induction can be regarded as an antiestrogenic mechanism, since P450 1A1 and P450 1B1 induce 2- and 4-hydroxylation of estrogens and thus limit E₂ concentrations and AhR activation mediates ubiquitination and degradation of ER α .⁴⁸

are stable biomarkers that reflect levels of the catechols, 2-OHE₁ and 4-OHE₁, which are produced by P450 1A1 and P450 1B1, respectively. Induction of the 2-hydroxylation pathway occurred in MCF-10A cells with little effect on the genotoxic 4-hydroxylation pathway.⁴⁸ Significant CYP1A1 induction was detected by RT-qPCR with only slight increases in CYP1B1 expression in both MCF-10A and MCF-7 cells. An analysis of the bioactive compounds showed that 6-PN was the most potent activator of AhR signaling, which induces CYP1A1 and CYP1B1 genes, although 8-PN did show some activity in MCF-7 cells.⁴⁸ Since ER is known to selectively suppress CYP1A1 expression, 6-PN, similar to other AhR agonists, likely also targets ER for proteasomal degradation, which leads to substantial CYP1A1 induction (Figure 5).⁴⁹

7. REACTIVE OXYGEN SPECIES AS A BIOLOGICAL TARGET

Hops contain many prenylated aromatic compounds, such as 8-PN and XH, that can act as scavengers of reactive oxygen species (ROS) and prevent oxidative damage to proteins and DNA.^{50–53} The data suggest that the prenylated phenols in particular can protect cellular proteins such as low-density lipoproteins (LDL) from oxidation.⁵³ Similar protective effects on DNA oxidation have been reported, especially for XH.^{54,55} XH could be an indirect antioxidant by increasing GSH. A small clinical study where participants consumed a drink containing XH (12 mg/day) showed a significant drop in 8-oxo-2'-deoxyguanosine (8-oxo-dG) levels, a biomarker of hydroxy radical induced oxidative DNA damage.⁵⁶ Beer has also been shown to protect DNA from oxidative damage and the amount of protection correlated with the content of phenolic constituents.⁵⁷ Finally, the antioxidant activities of a spent hop extract and the isolated hop compounds were measured according to their relative ability to scavenge DPPH free radicals.⁵⁵ The data showed that the hop extract and compounds had weak radical scavenging activities, which suggests other biological targets might be more important. At higher concentrations, pro-oxidant activities have also been reported for XH. In a study using breast cancer cells (MCF-7), XH decreased ROS production at low concentrations; however, XH increased ROS at higher concentrations.⁵¹

8. KEAP1-NRF2 AS A BIOLOGICAL TARGET (FIGURE 6)

XH has demonstrated various chemopreventive properties. For example, alkylation of Keap1 by electrophiles such as the α,β -unsaturated carbonyl on XH (Figure 1) causes accumulation of Nrf2 in the nucleus where it synthesizes detoxification enzymes such as NQO1, heme oxygenase-1 (HO-1), and GST (Figure 6).^{33,55,58,59} For example, it has been shown that

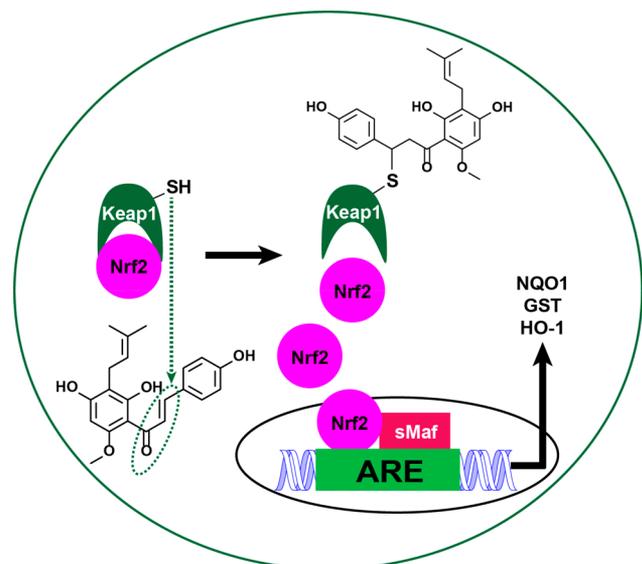


Figure 6. XH alkylation of cysteine residues in Keap1 leading to activation and translocation of Nrf2 to the nucleus inducing the synthesis of detoxification enzymes such as NQO1, HO-1, and GST.^{33,55}

pretreatment of rat derived pheochromocytoma (PC12) cells with XH at submicromolar concentrations upregulated a panel of detoxification genes as well as the corresponding gene

products, including GSH, HO-1, NQO1, thioredoxin, and thioredoxin reductase.⁵² Keap1 has been identified as a target protein of XH using a click chemistry approach as well as using a LC-MS/MS assay.^{60,61} A spent hop extract and XH have been demonstrated to induce the activity of NQO1 *in vitro* and *in vivo* animal models.³³ Induction of NQO1 by XH has been shown to reduce menadione induced DNA damage *in vitro*.⁵⁵ Similarly, XH protected DNA against the genotoxicity of 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), benzo(a)pyrene, and oxidative DNA damage in HepG2 cells, rat liver slices, and *in vivo*.^{62–65} In a human intervention trial where 22 subjects drank a drink containing 12 mg of XH, lymphocytes were collected and incubated with carcinogens and human liver homogenate (S9). The data showed a significant reduction in DNA damage and a clear induction of α -GST (43%), suggesting the Keap1-Nrf2 pathway was involved.⁶⁶

9. IKK/NF- κ B AS A BIOLOGICAL TARGET (FIGURE 7)

Another cytoprotective pathway of XH is the IKK/NF- κ B pathway, which leads to anti-inflammatory activities. NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) regulates genes involved in the formation of pro-inflammatory factors, such as cytokines, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2; Figure 7). As a result,

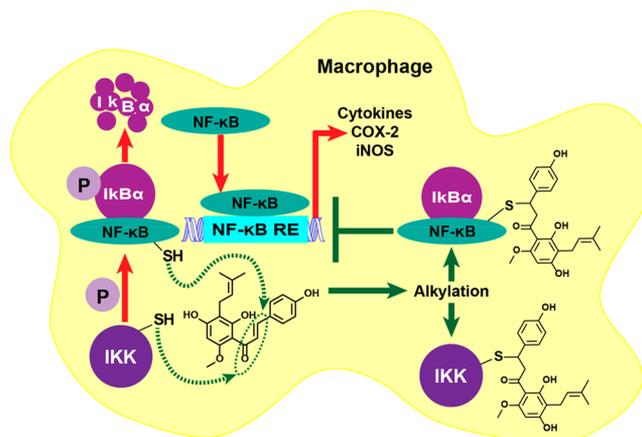


Figure 7. XH alkylation of IKK and NF- κ B cysteine residues. I κ B α sequesters NF- κ B in the cytoplasm, yet inflammatory stimuli activate IKK phosphorylation of I κ B α and subsequent I κ B α degradation. This allows NF- κ B to translocate to the nucleus and activate inflammatory genes, such as iNOS and COX-2. XH is anti-inflammatory, since it alkylates cysteine residues in IKK, which prevents degradation of I κ B α , and cysteine residues in NF- κ B, which prevents NF- κ B DNA binding.⁷⁰

inhibition of NF- κ B activation is a key chemopreventive target.^{3,67} XH has been shown to downregulate both constitutive and inducible NF- κ B activation.^{3,68,69} The mechanism likely involves modification of the cysteine residues by XH in I κ B α kinase (IKK) and/or NF- κ B, which directly leads to suppression of NF- κ B regulated gene products and potentiation of apoptosis in human leukemia and myeloma cells (Figure 7).⁷⁰ The presence of reducing agents reversed the effect of XH on IKK, suggesting a role for cysteine residues.⁷⁰ In support of this, XH had no effect on the IKK activity when cysteine residue 179 of IKK was mutated to alanine and DNA binding of the p65 subunit of NF- κ B was inhibited when cysteine residue 38 was mutated to serine.^{69,70} Further evidence from our laboratory has shown that a standardized spent hop extract

and XH can downregulate NF- κ B luciferase in MCF-7 cells (Figure 8) and inhibit iNOS activity in RAW 264.7 cells

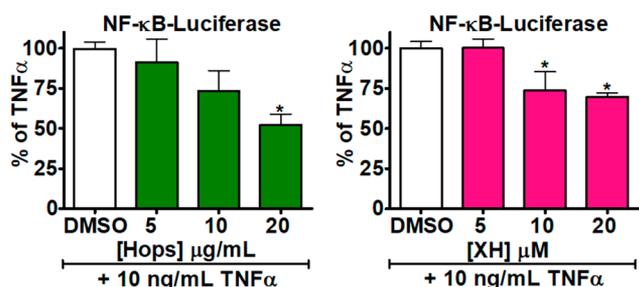


Figure 8. Hops and XH inhibition of NF- κ B luciferase. MCF-7 cells were grown in six-well plates (25×10^4 cells/well) for 24 h in estrogen-free media at 37 °C. After 24 h, cells were transfected with the NF- κ B-luciferase reporter plasmid and treated with TNF- α (10 ng/mL) and (A) the standardized spent hop extract or (B) XH for 6 h before lysis with passive lysis buffer. NF- κ B-luciferase activity was measured using the Promega Dual-Luciferase Reporter Assay System (Madison, WI). Results represent the mean \pm SD of three independent experiments analyzed by one-way ANOVA with Dunnett's multiple comparison post-test; * $p < 0.001$.

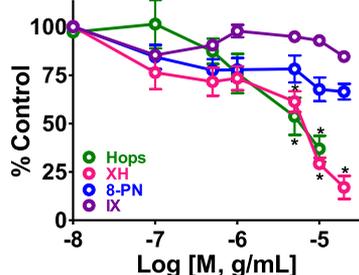


Figure 9. Hops and XH inhibition of iNOS. RAW 264.7 cells were plated at a density of 12×10^4 cells/mL in 96-well plates and incubated at 37 °C for 24 h. Cells were treated with LPS (1 μ g/mL) and (A) the standardized spent hop extract or (B) XH and incubated at 37 °C for 24 h. Griess reagent (150 μ L of 0.5% sulfanilamide and 0.05% (*N*-1-naphthyl) ethylenediamine dihydrochloride in 2.5% w/w H₃PO₄) was added to a 96-well plate containing media collected from cells (50 μ L). Nitrite levels were detected after the plate was incubated at RT for 30 min. Absorbance was measured at 530 nm, and concentrations were calculated using a NaNO₂ standard curve. Results represent the mean \pm SD of three independent experiments and are analyzed by one-way ANOVA with Dunnett's multiple comparison post-test; * $p < 0.001$.

(Figure 9). Similar data were obtained previously, which showed XH downregulated TNF α -induced iNOS activity.¹⁰ Additionally, XH inhibits the signal transducer and activator of transcription 3 (STAT3) and activators of this pathway (e.g., IL-6), which leads to reduced inflammatory response, survival, migration, and angiogenesis.⁷¹ The mechanism reportedly involves XH interfering with LPS receptor binding. In summary, several lines of evidence suggest that XH-mediated inhibition of pro-inflammatory biological targets can play a key role in inhibiting carcinogenesis.⁷¹

10. XH TARGETS BCL-2 AND MITOCHONDRIA LEADING TO APOPTOSIS (FIGURE 10)

While XH induces cytoprotective mechanism at low concentrations, it leads to apoptosis and cell cytotoxicity at higher concentrations.⁵¹ The apoptosis inducing and cell proliferation

inhibiting properties by XH have been demonstrated in several cell lines as well as in animal tumor models.^{7,72–76} Different pathways have been described for XH that lead ultimately to apoptosis and reduction of cell survival.^{73–76} For example, XH (15 μ M for 48 h) has been demonstrated to activate caspases of the extrinsic (caspase 8) and intrinsic pathway (caspase 9) in human colon cancer cells, resulting in downstream activation of caspase 3 and cleavage of Poly(ADP-ribose)polymerase (PARP), as the hallmark of apoptosis (Figure 10).⁷⁴ Downregulation of

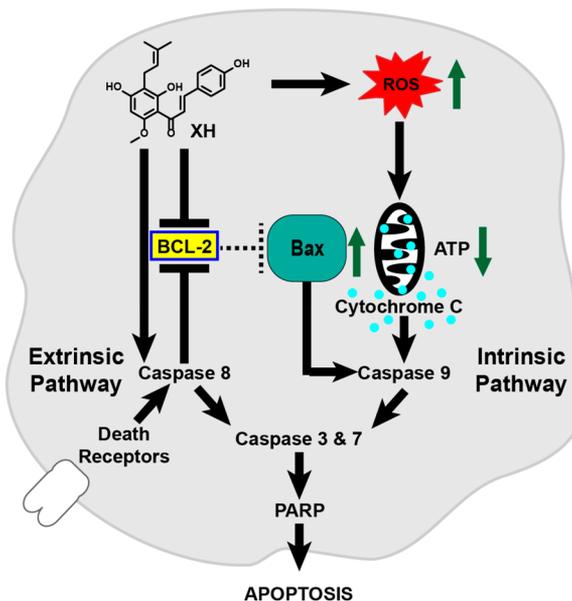


Figure 10. XH induction of apoptosis at high concentrations. XH treatment initiates intrinsic and extrinsic apoptosis pathways by multiple mechanisms, including downregulation of antiapoptotic (BCL-2) and induction of pro-apoptotic (Bax) proteins, increases in ROS, leading to decreased mitochondrial membrane potential and subsequent cytochrome C release, and activation of caspases. As a result, cleavage of PARP and reduced proliferation of tumor cells has been observed after XH treatments.^{72–75}

the antiapoptotic protein B-cell lymphoma 2 (Bcl-2) and upregulation of the pro-apoptotic protein Bcl-2-associated X protein (Bax) contributed to activation of the intrinsic apoptosis pathway.⁷⁴ In a different study, induction of oxidative stress mainly through superoxide formation in mitochondria has been reported as a cause for apoptosis induction by XH.⁷³ Further analysis showed a decrease in mitochondrial electron flux and intracellular ATP levels by XH (around 25 μ M), leading to cytochrome c release and ultimately to apoptosis (Figure 10). Similarly, in an analysis in adipocytes, XH increased ROS, resulting in a lower mitochondrial membrane potential, cytochrome C release, and induction of apoptosis.⁷² As mentioned above (see section 6, Reactive Oxygen Species as a Biological Target), at low concentrations, XH decreased ROS production in the breast cancer cell line, MCF-7, while XH increased ROS and reduced cell viability at higher concentrations.⁵¹ This is in line with other studies reporting that the Michael acceptor, XH, reduced oxidative stress and inflammation at low concentrations and leads to a decrease of cell proliferation at higher concentrations.^{9,55,77} As an example, XH has also been reported to decrease biomarkers of apoptosis and oxidative stress in an aged rat model of increased apoptosis, oxidative stress, and inflammation.⁷⁸

Studies of XH-rich hop extracts analyzing the influence on apoptosis remain preliminary. *In vitro* studies in endometrial cancer cells (Ishikawa) and mouse hepatoma cells (Hepa1c1c7) have shown that cell survival after treatment with various spent hop extracts is directly correlated with the XH concentration.⁸ NQO1 induction properties and estrogenic activities of these spent hop extracts were observed at much lower concentrations than a decrease in cell survival.⁸ Overall, these results may indicate that XH in hop products may more likely protect against oxidative stress *in vivo*, as XH concentrations that lead to apoptosis may be more difficult to reach after administration of clinical doses of (spent) hop extracts.

11. AMP-ACTIVATED PROTEIN KINASE SIGNALING PATHWAY (AMPK) AS A BIOLOGICAL TARGET OF XH TO REGULATE LIPID AND GLUCOSE METABOLISM (FIGURE 11)

Different *in vitro* and *in vivo* studies also demonstrated anti-obesity and antiglycemic effects for XH.^{79–82} Similarly, in a male rodent obesity model, an XH-rich hop extract significantly decreased body weight as well as liver triacylglycerol and cholesterol values compared to the high fat diet.⁸³ XH's influence on body weight, lipid metabolism, and glucose levels has been reported to be mediated through multiple biological targets.^{79–82} An increase of lipolysis and an accelerated breakdown of fatty acids through enhanced beta oxidation (Figure 11)

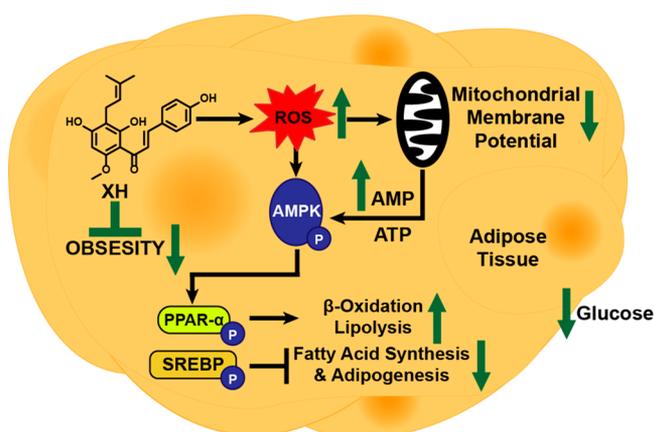


Figure 11. XH influence on lipid and glucose metabolism. XH exhibits antiobesity and antiglycemic effects by modulating the AMPK pathway. XH increases ROS, which can directly activate AMPK or decrease mitochondrial coupling, resulting in an increased AMP/ATP ratio, and in turn activate the AMPK pathway. AMPK phosphorylates multiple targets, including PPAR- α and SREBP, which results in increased β -oxidation and lipolysis and decreased fatty acid synthesis and adipogenesis.^{80–82}

has been described in adipocytes⁸¹ and in obesity animal models after treatment with XH.⁸² Beta oxidation is likely increased by XH through activation of the AMPK signaling pathway⁸¹ and subsequent increase of peroxisome proliferator-activated receptor α (PPAR α) mRNA expression (Figure 11).⁸²

AMPK is the principal sensor of cellular energy status and is activated by an increase of AMP/ATP ratio and ROS.^{84,85} The AMP/ATP ratio can be enhanced through mitochondrial impairment. Mitochondrial uncoupling (electron transport that is not used to drive ATP synthesis) induced by XH-mediated short-term ROS formation has been demonstrated to increase AMP-activated protein kinase signaling pathways,⁸⁰ leading to

increased catabolism and higher energy expenditure (Figure 11).⁸¹ An elevation of the AMPK signaling pathway by XH also led to downregulation of sterol regulatory element-binding protein-1c (SREBP-1c) mRNA expression in a rodent obesity model, consequently reducing fatty acid synthesis and adipogenesis.⁸²

In addition, XH has been reported to increase beige-ing of white adipocytes, which involves accelerated thermoregulation and lipolysis contributing to XH's antiobesity effects.⁸¹ A characteristic of beige adipocytes is enhanced mitochondrial biogenesis and thus higher energy expenditure. XH markedly increased mitochondrial content and markers of mitochondrial biogenesis in adipocytes, such as PGC-1 α .⁸¹ XH's influence on mitochondria and oxidative stress followed by AMPK signaling pathway induction may in part explain the observed antiobesity activity of XH in rodent obesity studies.⁸⁰ Interestingly, impaired energy metabolism is also a primary defect in type 2 diabetes, and two current diabetic therapeutics have been shown to act via AMPK activation.⁸⁵ As a result, stimulation of AMPK by XH might also explain some of its antiglycemic effects (Figure 11).⁸⁶

Other biological targets for XH's influence on lipid metabolism have been described. For example, XH has been reported to inhibit rat liver diacylglycerol acyltransferase and triglyceride transport, as well as to inhibit cholesteryl ester transfer protein activity and to induce apoptosis of mature adipocytes.⁷⁹ While different rodent obesity models in male animals reported antiobesity and antiglycemic effects after XH treatment, these activities were not reported in a similar model in female rats.⁸⁶ Additional studies are needed to elucidate the difference of XH treatment on sex-specific characteristics of lipid metabolism. Overall, available data suggest a regulating influence of XH and XH-rich hop extracts on lipid metabolism at least in male animals and warrants further analysis in clinical studies.

12. AMP-ACTIVATED PROTEIN KINASE SIGNALING PATHWAY (AMPK) AS A BIOLOGICAL TARGET OF XH TO REDUCE ANGIOGENESIS

The AMPK pathway seems also to be involved in XH's anti-angiogenic properties.⁸⁷ XH showed antiangiogenic effects in *in vitro* and *in vivo* models.^{68,87,88} Treatment of endothelial cells with XH increased AMPK activity, which likely resulted in antiangiogenic effects. XH-mediated AMPK activation resulted in a decrease of endothelial NOS phosphorylation, ultimately reducing NO levels and thus reducing angiogenesis. In addition, XH's antiangiogenic activity has also been shown to be mediated by inhibition of AKT and other pathways.^{68,87} XH-rich hop extracts have not yet been well analyzed for its anti-angiogenic activity; therefore, additional studies are warranted to elucidate the overall antiangiogenic properties of hop extracts.

13. INHIBITION OF P450S AS A BIOLOGICAL TARGET

Finally, the hop compounds have been reported to inhibit several P450 enzymes.^{89–91} A number of different beers have also been tested with significant inhibition observed with various P450s particularly with the dark porter style beers.^{92,93} At 10 μ M, XH almost completely inhibited the 7-ethoxyresorufin O-deethylase activity of P450 1A1 and P450 1B1.⁸⁹ In contrast, P450 1A2 was inhibited by 8-PN and IX (>90% inhibition, 10 μ M). P450 3A4 and P450 2E1 were not inhibited by the hop compounds, which shows they are selective inhibitors of P450s.⁸⁹ The standardized spent hop extract at 5 μ g/mL showed significant inhibition of P450 2C8 and P450 2C9.⁹⁰

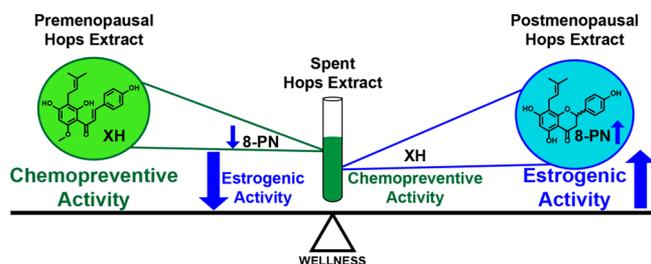


Figure 12. Designer extracts. Two spent hop extracts were designed with different concentrations of 8-PN. One extract with higher 8-PN concentrations modulates estrogenic effects in postmenopausal women, yet retains chemopreventive effects derived from XH. The second extract designated for use by younger women (premenopausal extract) contains lower concentrations of 8-PN than the postmenopausal extract but similar XH concentrations with potential chemopreventive properties.⁸

IX was the most potent inhibitor of P450 2C8, whereas 8-PN had highest inhibitory activity for P450 2C9.⁹⁰ Whether these hop flavonoids would lead to P450 inhibition after administration of the standardized spent hop extract in humans is currently under analysis in a phase I study in our Botanical Center.

14. DESIGNER HOP EXTRACTS SHOWED SIMULTANEOUS MODIFICATION OF TWO BIOLOGICAL TARGETS (FIGURE 12)

We recently have shown that hop extracts can be modified to selectively increase or decrease the concentrations of certain bioactive compounds in hops.⁸ For example, all populations of women would benefit from the chemopreventive compounds such as XH; however, premenopausal women are unlikely to benefit from the estrogenic constituent 8-PN. Through a series

of differential liquid-only chromatographic steps, two spent hop extracts were created: one designated for premenopausal women with significant amounts of XH and very little 8-PN, and one for postmenopausal women with XH and 8-PN for menopausal symptom relief (Figure 12). These data show that once the bioactive compounds have been identified in extracts, DESIGNER (Deplete and Enrich Select Ingredients to Generate Normalized Extract Resources) extracts could be created to maximize beneficial outcomes and limit potential unwanted effects.

15. CONCLUSIONS AND FUTURE DIRECTIONS

In conclusion, accumulating data are showing that spent hop extracts have multiple biological targets, leading to polypharmacological outcomes *in vivo* (Figure 13). Estrogenic effects associated with hops are most likely due to 8-PN, a potent ER ligand. This phytoestrogen is also an aromatase inhibitor that could protect women from estrogen carcinogenesis. The prenylated phenols are weak scavengers of ROS; however, these compounds might function indirectly as antioxidants through an increase of GSH synthesis and other antioxidative enzymes. As a Michael acceptor, XH is a Keap1-Nrf2 activator, leading to induction of detoxification enzymes such as NQO1 and GST. XH also alkylates IKK and NF- κ B, resulting in anti-inflammatory activity. At higher concentrations, XH can have pro-oxidant effects leading to apoptosis and a decrease of cell proliferation. Therefore, XH concentrations in the high nanomolar to low micromolar range would be ideal to achieve XH's cytoprotective effect *in vivo*. Finally, XH has been described to activate AMPK, which is at least in part responsible for XH's antiobesity and antiglycemic effects. Hops also contain 6-PN, a potent agonist of AhR, leading to selective

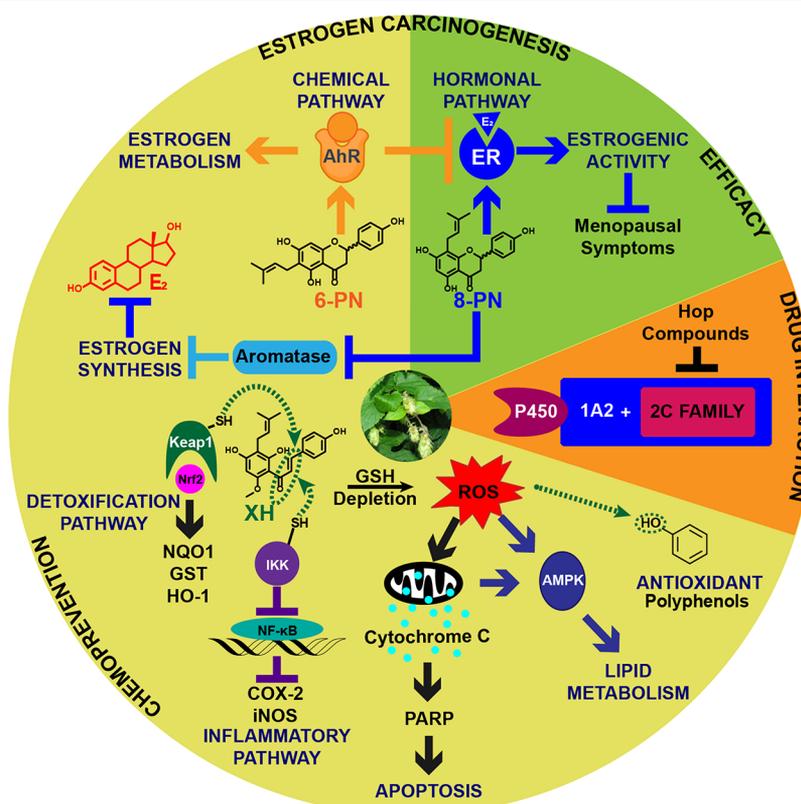


Figure 13. Summary of biological targets of spent hop extracts.

upregulation of the P450 1A1-mediated estrogen detoxification pathway. The hop compounds are also selective inhibitors of various P450s including P450 1A1, 1A2, 1B1, 2C8, 2C9, and 2C19. Finally, experiments with designer extracts have shown that spent hop extracts can be prepared which contain tailored doses of desirable bioactive compounds while limiting exposure to compounds that might be associated with adverse effects. Understanding the mechanism of action and biological targets of bioactive compounds in hop dietary supplements used for women's health will ultimately lead to standardized botanical products with higher efficacy and safety and help optimize the chemopreventive properties and other health benefits of hops.

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ABBREVIATIONS

AKR1C3, aldoketoreductase 1C3; AMPK, AMP-activated protein kinase; ARE, antioxidant response element; AhR, aryl hydrocarbon receptor; Bax, pro-apoptotic protein Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; COX-2, cyclooxygenase-2; DMX, desmethylxanthohumol; ER, estrogen receptor; GST, glutathione-S-transferase; GSH, glutathione; HO-1, heme oxygenase-1; IKK, inhibitor of kappa B protein kinase; iNOS, inducible nitric oxide synthase; IX, isoxanthohumol; Keap1, Kelch like-ECH-associated protein 1; 2-MeOE₁, 2-methoxyestrone; 4-MeOE₁, 4-methoxyestrone; NQO1, NAD(P)H-quinone oxidoreductase 1; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; Nrf2, nuclear factor (erythroid-derived 2)-like 2; PPAR α , peroxisome proliferator-activated receptor α ; PARP, poly(ADP-ribose)-polymerase; 6-PN, 6-prenylningenin; 8-PN, 8-prenylningenin; ROS, reactive oxygen species; SREBP-1c, sterol regulatory element-binding protein-1c; SFE, supercritical fluid extraction; TNF α , tumor necrosis factor alpha; XH, xanthohumol

REFERENCES

(1) Stevens, J. F., and Page, J. E. (2004) Xanthohumol and related prenylflavonoids from hops and beer: to your good health! *Phytochemistry* 65, 1317–1330.

(2) Karabin, M., Hudcova, T., Jelinek, L., and Dostalek, P. (2016) Biologically active compounds from hops and prospects for their use. *Compr. Rev. Food Sci. Food Saf.* 15, 542–567.

(3) Karabin, M., Hudcova, T., Jelinek, L., and Dostalek, P. (2015) Biotransformations and biological activities of hop flavonoids. *Biotechnol. Adv.* 33, 1063–1090.

(4) Aghamiri, V., Mirghafourvand, M., Mohammad-Alizadeh-Charandabi, S., and Nazemiyeh, H. (2016) The effect of Hop (*Humulus lupulus* L.) on early menopausal symptoms and hot flashes: A randomized placebo-controlled trial. *Complement. Ther. Clin. Pract.* 23, 130–135.

(5) Dietz, B. M., Hajirahimkhan, A., Dunlap, T. L., and Bolton, J. L. (2016) Botanicals and their bioactive phytochemicals for women's health. *Pharmacol. Rev.* 68, 1026–1073.

(6) Keiler, A. M., Zierau, O., and Kretzschmar, G. (2013) Hop extracts and hop substances in treatment of menopausal complaints. *Planta Med.* 79, 576–579.

(7) Venturelli, S., Burkard, M., Biendl, M., Lauer, U. M., Frank, J., and Busch, C. (2016) Prenylated chalcones and flavonoids for the prevention and treatment of cancer. *Nutrition* 32, 1171–1178.

(8) Dietz, B. M., Chen, S. N., Alvarenga, R. F. R., Dong, H., Nikolic, D., Biendl, M., van Breemen, R. B., Bolton, J. L., and Pauli, G. F. (2017) DESIGNER extracts as tools to balance estrogenic and chemopreventive activities of botanicals for women's health. *J. Nat. Prod.* 80, 2284–2294.

(9) Gerhäuser, C., Alt, A., Heiss, E., Gamal-Eldeen, A., Klimo, K., Knauff, J., Neumann, I., Scherf, H. R., Frank, N., Bartsch, H., and Becker, H. (2002) Cancer chemopreventive activity of xanthohumol, a natural product derived from hop. *Mol. Cancer Ther.* 1, 959–969.

(10) Cho, Y. C., Kim, H. J., Kim, Y. J., Lee, K. Y., Choi, H. J., Lee, I. S., and Kang, B. Y. (2008) Differential anti-inflammatory pathway by xanthohumol in IFN-gamma and LPS-activated macrophages. *Int. Immunopharmacol.* 8, 567–573.

(11) Chadwick, L. R., Nikolic, D., Burdette, J. E., Overk, C. R., Bolton, J. L., van Breemen, R. B., Fröhlich, R., Fong, H. H., Farnsworth, N. R., and Pauli, G. F. (2004) Estrogens and congeners from spent hops (*Humulus lupulus*). *J. Nat. Prod.* 67, 2024–2032.

(12) Chadwick, L. R., Pauli, G. F., and Farnsworth, N. R. (2006) The pharmacognosy of *Humulus lupulus* L. (hops) with an emphasis on estrogenic properties. *Phytomedicine* 13, 119–131.

(13) Kötter, U., and Biendl, M. (2010) Hops (*Humulus lupulus*): A Review of its Historic and Medicinal Uses. *HerbalGram* 87, 44–57.

(14) Dresel, M., Dunkel, A., and Hofmann, T. (2015) Sensomics analysis of key bitter compounds in the hard resin of hops (*Humulus lupulus* L.) and their contribution to the bitter profile of Pilsner-type beer. *J. Agric. Food Chem.* 63, 3402–3418.

(15) Milligan, S., Kalita, J., Pocock, V., Heyerick, A., De Cooman, L., Rong, H., and De Keukeleire, D. (2002) Oestrogenic activity of the hop phyto-oestrogen, 8-prenylningenin. *Reproduction* 123, 235–242.

(16) Chen, S. N., Lankin, D. C., Chadwick, L. R., Jaki, B. U., and Pauli, G. F. (2009) Dynamic residual complexity of natural products by qHNMR: solution stability of desmethylxanthohumol. *Planta Med.* 75, 757–762.

(17) Nikolic, D., Li, Y., Chadwick, L. R., and van Breemen, R. B. (2006) In vitro studies of intestinal permeability and hepatic and intestinal metabolism of 8-prenylningenin, a potent phytoestrogen from hops (*Humulus lupulus* L.). *Pharm. Res.* 23, 864–872.

(18) Pang, Y., Nikolic, D., Zhu, D., Chadwick, L. R., Pauli, G. F., Farnsworth, N. R., and van Breemen, R. B. (2007) Binding of the hop (*Humulus lupulus* L.) chalcone xanthohumol to cytosolic proteins in Caco-2 intestinal epithelial cells. *Mol. Nutr. Food Res.* 51, 872–879.

(19) Yilmazer, M., Stevens, J. F., and Buhler, D. R. (2001) In vitro glucuronidation of xanthohumol, a flavonoid in hop and beer, by rat and human liver microsomes. *FEBS Lett.* 491, 252–256.

(20) Legette, L., Karnpracha, C., Reed, R. L., Choi, J., Bobe, G., Christensen, J. M., Rodriguez-Proteau, R., Purnell, J. Q., and Stevens, J. F. (2014) Human pharmacokinetics of xanthohumol, an

antihyperglycemic flavonoid from hops. *Mol. Nutr. Food Res.* 58, 248–255.

(21) van Breemen, R. B., Yuan, Y., Banuvar, S., Shulman, L. P., Qiu, X., Alvarenga, R. F., Chen, S. N., Dietz, B. M., Bolton, J. L., Pauli, G. F., Krause, E., Viana, M., and Nikolic, D. (2014) Pharmacokinetics of prenylated hop phenols in women following oral administration of a standardized extract of hops. *Mol. Nutr. Food Res.* 58, 1962–1969.

(22) Bolca, S., Li, J., Nikolic, D., Roche, N., Blondeel, P., Possemiers, S., De Keukeleire, D., Bracke, M., Heyerick, A., van Breemen, R. B., and Depypere, H. (2010) Disposition of hop prenylflavonoids in human breast tissue. *Mol. Nutr. Food Res.* 54 (Suppl 2), S284–S294.

(23) Rad, M., Hümpel, M., Schaefer, O., Schoemaker, R. C., Schleuning, W. D., Cohen, A. F., and Burggraaf, J. (2006) Pharmacokinetics and systemic endocrine effects of the phytoestrogen 8-prenylnaringenin after single oral doses to postmenopausal women. *Br. J. Clin. Pharmacol.* 62, 288–296.

(24) Martinez, S. E., and Davies, N. M. (2015) Enantiospecific pharmacokinetics of isoxanthohumol and its metabolite 8-prenylnaringenin in the rat. *Mol. Nutr. Food Res.* 59, 1674–1689.

(25) Guo, J., Nikolic, D., Chadwick, L. R., Pauli, G. F., and van Breemen, R. B. (2006) Identification of human hepatic cytochrome P450 enzymes involved in the metabolism of 8-prenylnaringenin and isoxanthohumol from hops (*Humulus lupulus* L.). *Drug Metab. Dispos.* 34, 1152–1159.

(26) Possemiers, S., Rabot, S., Espin, J. C., Bruneau, A., Philippe, C., Gonzalez-Sarrias, A., Heyerick, A., Tomas-Barberan, F. A., De Keukeleire, D., and Verstraete, W. (2008) Eubacterium limosum activates isoxanthohumol from hops (*Humulus lupulus* L.) into the potent phytoestrogen 8-prenylnaringenin in vitro and in rat intestine. *J. Nutr.* 138, 1310–1316.

(27) Calvo-Castro, L. A., Burkard, M., Sus, N., Scheubeck, G., Leischner, C., Lauer, U. M., Bony-Westphal, A., Hund, V., Busch, C., Venturelli, S., and Frank, J. (2018) The Oral Bioavailability of 8-Prenylnaringenin from Hops (*Humulus Lupulus* L.) in Healthy Women and Men is Significantly Higher than that of its Positional Isomer 6-Prenylnaringenin in a Randomized Crossover Trial. *Mol. Nutr. Food Res.* 62, No. 1700838.

(28) Overk, C. R., Yao, P., Chadwick, L. R., Nikolic, D., Sun, Y., Cuendet, M. A., Deng, Y., Hedayat, A. S., Pauli, G. F., Farnsworth, N. R., van Breemen, R. B., and Bolton, J. L. (2005) Comparison of the in vitro estrogenic activities of compounds from hops (*Humulus lupulus*) and red clover (*Trifolium pratense*). *J. Agric. Food Chem.* 53, 6246–6253.

(29) Helle, J., Kraker, K., Bader, M. I., Keiler, A. M., Zierau, O., Vollmer, G., Welsh, J., and Kretzschmar, G. (2014) Assessment of the proliferative capacity of the flavanones 8-prenylnaringenin, 6-(1,1-dimethylallyl)naringenin and naringenin in MCF-7 cells and the rat mammary gland. *Mol. Cell. Endocrinol.* 392, 125–135.

(30) Bowe, J., Li, X. F., Kinsey-Jones, J., Heyerick, A., Brain, S., Milligan, S., and O'Byrne, K. (2006) The hop phytoestrogen, 8-prenylnaringenin, reverses the ovariectomy-induced rise in skin temperature in an animal model of menopausal hot flashes. *J. Endocrinol.* 191, 399–405.

(31) Stulikova, K., Karabin, M., Nesporek, J., and Dostalek, P. (2018) Therapeutic perspectives of 8-prenylnaringenin, a potent phytoestrogen from hops. *Molecules* 23, 660.

(32) Nikolic, D., and van Breemen, R. B. (2013) Analytical methods for quantitation of prenylated flavonoids from hops. *Curr. Anal. Chem.* 9, 71–85.

(33) Dietz, B. M., Hagos, G. K., Eskra, J. N., Wijewickrama, G. T., Anderson, J. R., Nikolic, D., Guo, J., Wright, B., Chen, S. N., Pauli, G. F., van Breemen, R. B., and Bolton, J. L. (2013) Differential regulation of detoxification enzymes in hepatic and mammary tissue by hops (*Humulus lupulus*) in vitro and in vivo. *Mol. Nutr. Food Res.* 57, 1055–1066.

(34) Overk, C. R., Guo, J., Chadwick, L. R., Lantvit, D. D., Minassi, A., Appendino, G., Chen, S. N., Lankin, D. C., Farnsworth, N. R., Pauli, G. F., van Breemen, R. B., and Bolton, J. L. (2008) In vivo estrogenic comparisons of *Trifolium pratense* (red clover) *Humulus*

lupulus (hops), and the pure compounds isoxanthohumol and 8-prenylnaringenin. *Chem.-Biol. Interact.* 176, 30–39.

(35) Liu, J., Burdette, J. E., Xu, H., Gu, C., van Breemen, R. B., Bhat, K. P., Booth, N., Constantinou, A. I., Pezzuto, J. M., Fong, H. H., Farnsworth, N. R., and Bolton, J. L. (2001) Evaluation of estrogenic activity of plant extracts for the potential treatment of menopausal symptoms. *J. Agric. Food Chem.* 49, 2472–2479.

(36) Hajirahimkhan, A., Simmler, C., Yuan, Y., Anderson, J. R., Chen, S. N., Nikolic, D., Dietz, B. M., Pauli, G. F., van Breemen, R. B., and Bolton, J. L. (2013) Evaluation of estrogenic activity of licorice species in comparison with Hops used in botanicals for menopausal symptoms. *PLoS One* 8, No. e67947.

(37) Pisha, E., and Pezzuto, J. M. (1997) Cell-based assay for the determination of estrogenic and anti-estrogenic activities. *Methods Cell Sci.* 19, 37–43.

(38) Diel, P., Thomae, R. B., Caldarelli, A., Zierau, O., Kolba, S., Schmidt, S., Schwab, P., Metz, P., and Vollmer, G. (2004) Regulation of gene expression by 8-prenylnaringenin in uterus and liver of Wistar rats. *Planta Med.* 70, 39–44.

(39) Kretzschmar, G., Zierau, O., Wober, J., Tischer, S., Metz, P., and Vollmer, G. (2010) Prenylation has a compound specific effect on the estrogenicity of naringenin and genistein. *J. Steroid Biochem. Mol. Biol.* 118, 1–6.

(40) Keiler, A. M., Helle, J., Bader, M. I., Ehrhardt, T., Nestler, K., Kretzschmar, G., Bernhardt, R., Vollmer, G., Nikolic, D., Bolton, J. L., Pauli, G. F., Chen, S. N., Dietz, B. M., van Breemen, R. B., and Zierau, O. (2017) A standardized *Humulus lupulus* (L.) ethanol extract partially prevents ovariectomy-induced bone loss in the rat without induction of adverse effects in the uterus. *Phytomedicine* 34, 50–58.

(41) Keiler, A. M., Macejova, D., Dietz, B. M., Bolton, J. L., Pauli, G. F., Chen, S. N., van Breemen, R. B., Nikolic, D., Goerl, F., Muders, M. H., Zierau, O., and Vollmer, G. (2017) Evaluation of estrogenic potency of a standardized hops extract on mammary gland biology and on MNU-induced mammary tumor growth in rats. *J. Steroid Biochem. Mol. Biol.* 174, 234–241.

(42) Balunas, M. J., Su, B., Brueggemeier, R. W., and Kinghorn, A. D. (2008) Natural products as aromatase inhibitors. *Anti-Cancer Agents Med. Chem.* 8, 646–682.

(43) Adams, L. S., and Chen, S. (2009) Phytochemicals for breast cancer prevention by targeting aromatase. *Front. Biosci., Landmark Ed.* 14, 3846–3863.

(44) Nielsen, A. J., and McNulty, J. (2018) Polyphenolic natural products and natural product-inspired steroidal mimics as aromatase inhibitors. *Med. Res. Rev.*, DOI: 10.1002/med.21536.

(45) Monteiro, R., Faria, A., Azevedo, I., and Calhau, C. (2007) Modulation of breast cancer cell survival by aromatase inhibiting hop (*Humulus lupulus* L.) flavonoids. *J. Steroid Biochem. Mol. Biol.* 105, 124–130.

(46) Monteiro, R., Becker, H., Azevedo, I., and Calhau, C. (2006) Effect of hop (*Humulus lupulus* L.) flavonoids on aromatase (estrogen synthase) activity. *J. Agric. Food Chem.* 54, 2938–2943.

(47) Chen, S., Zhang, F., Sherman, M. A., Kijima, I., Cho, M., Yuan, Y. C., Toma, Y., Osawa, Y., Zhou, D., and Eng, E. T. (2003) Structure-function studies of aromatase and its inhibitors: a progress report. *J. Steroid Biochem. Mol. Biol.* 86, 231–237.

(48) Wang, S., Dunlap, T. L., Howell, C. E., Mbachu, O. C., Rue, E. A., Phansalkar, R., Chen, S. N., Pauli, G. F., Dietz, B. M., and Bolton, J. L. (2016) Hop (*Humulus lupulus* L.) extract and 6-prenylnaringenin induce P450 1A1 catalyzed estrogen 2-hydroxylation. *Chem. Res. Toxicol.* 29, 1142–1150.

(49) Marques, M., Laflamme, L., and Gaudreau, L. (2013) Estrogen receptor alpha can selectively repress dioxin receptor-mediated gene expression by targeting DNA methylation. *Nucleic Acids Res.* 41, 8094–8106.

(50) Zhang, X. L., Zhang, Y. D., Wang, T., Guo, H. Y., Liu, Q. M., and Su, H. X. (2014) Evaluation on antioxidant effect of xanthohumol by different antioxidant capacity analytical methods. *J. Chem.* 2014, 249485.

- (51) Blanquer-Rossello, M. M., Oliver, J., Valle, A., and Roca, P. (2013) Effect of xanthohumol and 8-prenylnaringenin on MCF-7 breast cancer cells oxidative stress and mitochondrial complexes expression. *J. Cell. Biochem.* 114, 2785–2794.
- (52) Yao, J., Zhang, B., Ge, C., Peng, S., and Fang, J. (2015) Xanthohumol, a polyphenol chalcone present in hops, activating Nrf2 enzymes to confer protection against oxidative damage in PC12 cells. *J. Agric. Food Chem.* 63, 1521–1531.
- (53) Miranda, C. L., Stevens, J. F., Ivanov, V., McCall, M., Frei, B., Deinzer, M. L., and Buhler, D. R. (2000) Antioxidant and prooxidant actions of prenylated and nonprenyated chalcones and flavanones in vitro. *J. Agric. Food Chem.* 48, 3876–3884.
- (54) Carvalho, D. O., Oliveira, R., Johansson, B., and Guido, L. F. (2016) Dose-dependent protective and inductive effects of xanthohumol on oxidative DNA damage in *Saccharomyces cerevisiae*. *Food Technol. Biotechnol.* 54, 60–69.
- (55) Dietz, B. M., Kang, Y. H., Liu, G., Eggler, A. L., Yao, P., Chadwick, L. R., Pauli, G. F., Farnsworth, N. R., Mesecar, A. D., van Breemen, R. B., and Bolton, J. L. (2005) Xanthohumol isolated from *Humulus lupulus* Inhibits menadione-induced DNA damage through induction of quinone reductase. *Chem. Res. Toxicol.* 18, 1296–1305.
- (56) Ferk, F., Mišik, M., Nersesyan, A., Pichler, C., Jäger, W., Szekeres, T., Marculescu, R., Poulsen, H. E., Henriksen, T., Bono, R., Romanazzi, V., Al-Serori, H., Biendl, M., Wagner, K. H., Kundi, M., and Knasmüller, S. (2016) Impact of xanthohumol (a prenylated flavonoid from hops) on DNA stability and other health-related biochemical parameters: Results of human intervention trials. *Mol. Nutr. Food Res.* 60, 773–786.
- (57) Rivero, D., Perez-Magarino, S., Gonzalez-Sanjose, M. L., Valls-Belles, V., Codoner, P., and Muniz, P. (2005) Inhibition of induced DNA oxidative damage by beers: correlation with the content of polyphenols and melanoidins. *J. Agric. Food Chem.* 53, 3637–3642.
- (58) Krajka-Kuźniak, V., Paluszczak, J., and Baer-Dubowska, W. (2013) Xanthohumol induces phase II enzymes via Nrf2 in human hepatocytes in vitro. *Toxicol. In Vitro* 27, 149–156.
- (59) Lee, I. S., Lim, J., Gal, J., Kang, J. C., Kim, H. J., Kang, B. Y., and Choi, H. J. (2011) Anti-inflammatory activity of xanthohumol involves heme oxygenase-1 induction via NRF2-ARE signaling in microglial BV2 cells. *Neurochem. Int.* 58, 153–160.
- (60) Brodziak-Jarosz, L., Fujikawa, Y., Pastor-Flores, D., Kasicki, S., Jirásek, P., Pitzl, S., Owen, R. W., Klika, K. D., Gerhäuser, C., Amslinger, S., and Dick, T. P. (2016) A click chemistry approach identifies target proteins of xanthohumol. *Mol. Nutr. Food Res.* 60, 737–748.
- (61) Liu, G., Eggler, A. L., Dietz, B. M., Mesecar, A. D., Bolton, J. L., Pezzuto, J. M., and van Breemen, R. B. (2005) Screening method for the discovery of potential cancer chemoprevention agents based on mass spectrometric detection of alkylated Keap1. *Anal. Chem.* 77, 6407–6414.
- (62) Plazar, J., Žegura, B., Lah, T. T., and Filipič, M. (2007) Protective effects of xanthohumol against the genotoxicity of benzo(a)pyrene (BaP), 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) and tert-butyl hydroperoxide (t-BOOH) in HepG2 human hepatoma cells. *Mutat. Res., Genet. Toxicol. Environ. Mutagen.* 632, 1–8.
- (63) Plazar, J., Filipič, M., and Groothuis, G. M. (2008) Antigenotoxic effect of Xanthohumol in rat liver slices. *Toxicol. In Vitro* 22, 318–327.
- (64) Viegas, O., Zegura, B., Pezdric, M., Novak, M., Ferreira, I. M., Pinho, O., and Filipič, M. (2012) Protective effects of xanthohumol against the genotoxicity of heterocyclic aromatic amines MeIQx and PhIP in bacteria and in human hepatoma (HepG2) cells. *Food Chem. Toxicol.* 50, 949–955.
- (65) Ferk, F., Huber, W. W., Filipič, M., Bichler, J., Haslinger, E., Mišik, M., Nersesyan, A., Grasl-Kraupp, B., Žegura, B., and Knasmüller, S. (2010) Xanthohumol, a prenylated flavonoid contained in beer, prevents the induction of preneoplastic lesions and DNA damage in liver and colon induced by the heterocyclic aromatic amine amino-3-methyl-imidazo[4,5-f]quinoline (IQ). *Mutat. Res., Fundam. Mol. Mech. Mutagen.* 691, 17–22.
- (66) Pichler, C., Ferk, F., Al-Serori, H., Huber, W., Jäger, W., Waldherr, M., Mišik, M., Kundi, M., Nersesyan, A., Herbacek, I., and Knasmüller, S. (2017) Xanthohumol prevents DNA damage by dietary carcinogens: results of a human intervention trial. *Cancer Prev. Res.* 10, 153–160.
- (67) Bharti, A. C., and Aggarwal, B. B. (2002) Nuclear factor-kappa B and cancer: its role in prevention and therapy. *Biochem. Pharmacol.* 64, 883–888.
- (68) Monteiro, R., Calhau, C., Silva, A. O., Pinheiro-Silva, S., Guerreiro, S., Gartner, F., Azevedo, I., and Soares, R. (2008) Xanthohumol inhibits inflammatory factor production and angiogenesis in breast cancer xenografts. *J. Cell. Biochem.* 104, 1699–1707.
- (69) Yadav, V. R., Prasad, S., Sung, B., and Aggarwal, B. B. (2011) The role of chalcones in suppression of NF-kappaB-mediated inflammation and cancer. *Int. Immunopharmacol.* 11, 295–309.
- (70) Harikumar, K. B., Kunnumakara, A. B., Ahn, K. S., Anand, P., Krishnan, S., Guha, S., and Aggarwal, B. B. (2009) Modification of the cysteine residues in I-kappaB kinase and NF-kappaB (p65) by xanthohumol leads to suppression of NF-kappaB-regulated gene products and potentiation of apoptosis in leukemia cells. *Blood* 113, 2003–2013.
- (71) Jiang, C. H., Sun, T. L., Xiang, D. X., Wei, S. S., and Li, W. Q. (2018) Anticancer activity and mechanism of xanthohumol: a prenylated flavonoid from hops (*Humulus lupulus* L.). *Front. Pharmacol.* 9, 530.
- (72) Yang, J. Y., Della-Fera, M. A., Rayalam, S., and Baile, C. A. (2007) Effect of xanthohumol and isoxanthohumol on 3T3-L1 cell apoptosis and adipogenesis. *Apoptosis* 12, 1953–1963.
- (73) Strathmann, J., Klimo, K., Sauer, S. W., Okun, J. G., Prehn, J. H., and Gerhäuser, C. (2010) Xanthohumol-induced transient superoxide anion radical formation triggers cancer cells into apoptosis via a mitochondria-mediated mechanism. *FASEB J.* 24, 2938–2950.
- (74) Pan, L., Becker, H., and Gerhäuser, C. (2005) Xanthohumol induces apoptosis in cultured 40–16 human colon cancer cells by activation of the death receptor- and mitochondrial pathway. *Mol. Nutr. Food Res.* 49, 837–843.
- (75) Guo, D., Zhang, B., Liu, S., and Jin, M. (2018) Xanthohumol induces apoptosis via caspase activation, regulation of Bcl-2, and inhibition of PI3K/Akt/mTOR-kinase in human gastric cancer cells. *Biomed. Pharmacother.* 106, 1300–1306.
- (76) Sun, Z., Zhou, C., Liu, F., Zhang, W., Chen, J., Pan, Y., Ma, L., Liu, Q., Du, Y., Yang, J., and Wang, Q. (2017) Inhibition of breast cancer cell survival by Xanthohumol via modulation of the Notch signaling pathway in vivo and in vitro. *Oncol. Lett.* 15, 908–916.
- (77) Dietz, B. M., and Bolton, J. L. (2011) Biological reactive intermediates (BRIs) formed from botanical dietary supplements. *Chem.-Biol. Interact.* 192, 72–80.
- (78) Fernandez-Garcia, C., Rancan, L., Paredes, S. D., Montero, C., de la Fuente, M., Vara, E., and Tresguerres, J. A. F. (2018) Xanthohumol exerts protective effects in liver alterations associated with aging. *Eur. J. Nutr.*, DOI: 10.1007/s00394-018-1657-6.
- (79) Dostalek, P., Karabin, M., and Jelinek, L. (2017) Hop Phytochemicals and Their Potential Role in Metabolic Syndrome Prevention and Therapy. *Molecules* 22, 1761.
- (80) Kirkwood, J. S., Legette, L. L., Miranda, C. L., Jiang, Y., and Stevens, J. F. (2013) A metabolomics-driven elucidation of the anti-obesity mechanisms of xanthohumol. *J. Biol. Chem.* 288, 19000–19013.
- (81) Samuels, J. S., Shashidharamurthy, R., and Rayalam, S. (2018) Novel anti-obesity effects of beer hops compound xanthohumol: role of AMPK signaling pathway. *Nutr. Metab.* 15, 42.
- (82) Takahashi, K., and Osada, K. (2017) Effect of Dietary Purified Xanthohumol from Hop (*Humulus lupulus* L.) Pomace on Adipose Tissue Mass, Fasting Blood Glucose Level, and Lipid Metabolism in KK-Ay Mice. *J. Oleo Sci.* 66, 531–541.

(83) Yui, K., Kiyofuji, A., and Osada, K. (2014) Effects of Xanthohumol-rich Extract from the Hop on Fatty Acid Metabolism in Rats Fed a High-fat Diet. *J. Oleo Sci.* 63, 159–168.

(84) Cardaci, S., Filomeni, G., and Ciriolo, M. R. (2012) Redox implications of AMPK-mediated signal transduction beyond energetic clues. *J. Cell Sci.* 125, 2115–2125.

(85) Shaw, R. J., Kosmatka, M., Bardeesy, N., Hurley, R. L., Witters, L. A., DePinho, R. A., and Cantley, L. C. (2004) The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. *Proc. Natl. Acad. Sci. U. S. A.* 101, 3329–3335.

(86) Legette, L. L., Moreno Luna, A. Y., Reed, R. L., Miranda, C. L., Bobe, G., Proteau, R. R., and Stevens, J. F. (2013) Xanthohumol lowers body weight and fasting plasma glucose in obese male Zucker fa/fa rats. *Phytochemistry* 91, 236–241.

(87) Gallo, C., Dallaglio, K., Bassani, B., Rossi, T., Rossello, A., Noonan, D. M., D'Uva, G., Bruno, A., and Albini, A. (2016) Hop derived flavonoid xanthohumol inhibits endothelial cell functions via AMPK activation. *Oncotarget* 7, 59917–59931.

(88) Rudzitis-Auth, J., Korbel, C., Scheuer, C., Menger, M. D., and Laschke, M. W. (2012) Xanthohumol inhibits growth and vascularization of developing endometriotic lesions. *Hum. Reprod.* 27, 1735–1744.

(89) Henderson, M. C., Miranda, C. L., Stevens, J. F., Deinzer, M. L., and Buhler, D. R. (2000) In vitro inhibition of human P450 enzymes by prenylated flavonoids from hops, *Humulus lupulus*. *Xenobiotica* 30, 235–251.

(90) Yuan, Y., Qiu, X., Nikolic, D., Chen, S. N., Huang, K., Li, G., Pauli, G. F., and van Breemen, R. B. (2014) Inhibition of human cytochrome P450 enzymes by hops (*Humulus lupulus*) and hop prenylphenols. *Eur. J. Pharm. Sci.* 53, 55–61.

(91) Dong, J., Zhang, Q., Cui, Q., Huang, G., Pan, X., and Li, S. (2016) Flavonoids and naphthoflavonoids: wider roles in the modulation of cytochrome P450 family 1 enzymes. *ChemMedChem* 11, 2102–2118.

(92) Foster, B. C., Kearns, N., Arnason, J. T., Saleem, A., Ogradowczyk, C., and Desjardins, S. (2009) Comparative study of hop-containing products on human cytochrome p450-mediated metabolism. *J. Agric. Food Chem.* 57, 5100–5105.

(93) Foster, B. C., Arnason, J. T., Saleem, A., Tam, T. W., Liu, R., Mao, J., and Desjardins, S. (2011) Comparative study of hops-containing products on human cytochrome P450-mediated metabolism. *J. Agric. Food Chem.* 59, 5159–5163.