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Evaluation of estrogenic potency of a standardized hops extract on mammary gland biology and on MNU-induced mammary tumor growth in rats

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Abstract

Supplements with estrogenic activities are intensively investigated as potential alternatives for the treatment of menopausal symptoms. These investigations include studies on their safety regarding potential breast cancer risks. Therefore, the aim of this study was to assess whether or not a standardized hops (*Humulus lupulus*) extract, containing 0.42% of the estrogenic flavanone, 8-prenylnaringenin, would stimulate growth of methyl-nitrosourea (MNU) induced mammary cancer in ovariectomized (OVX) Sprague-Dawley (SD) rats or would impact on the proliferative activity within the normal mammary gland of Wistar rats. To induce tumorigenesis SD-rats received an intraperitoneal injection of 50 mg/kg body weight of MNU on postnatal days PND 50 and 52. 28 days later animals were OVX or were SHAM operated (positive control) and randomly allocated and maintained for 140 days on either a phytoestrogen-free placebo diet (SHAM and negative control) or on the hops fortified diet. For the investigations in the normal mammary gland young adult Wistar rats were bilaterally OVX and randomly allocated to a control group fed to a phytoestrogen-free diet, or to a diet supplemented either with E₂-benzoate or the hops extract. As a major result, the tumor incidence was 15% (3 tumors totally) in OVX controls, whereas it was 85% (39 tumors totally) in SHAM operated positive controls. No tumors were detectable in the hops group. In addition, no estrogenic activity of the hops extract was detectable in uterus and

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liver of these animals. In investigations on the normal mammary gland, no impact of hops extract on the expression of estrogen dependent proliferation markers or of progesterone receptor became apparent. In conclusion, the lack of growth stimulation of MNU-induced breast cancer in OVX SD-rats and the lack of stimulation proliferative events in the normal mammary gland of OVX Wistar rats by standardized hops extracts provides an important piece of evidence regarding the safety of these extracts in the management of menopausal symptoms.

Keywords

Hops extract; Menopause; Estrogenicity; Breast cancer; Mammary gland

1. Introduction

Hormone Therapy (HT) was the treatment of choice to alleviate menopausal symptoms prior to the publication of the womens health initiative study in 2002, which reported increased risks for the development of hormone dependent cancers, cardiovascular problems, and stroke among women taking HT [1]. The long term follow-up of this study and studies performed later came to more moderate conclusions regarding the above mentioned risks in some study arms. However, breast cancer risk within the estrogen plus progesterone arm still persists even in these revisited or new studies [2,3]. Fear of breast cancer is one reason why many women all over the world have turned to botanical preparations to alleviate their symptoms [4]

There is a large variety of botanical products available on the market, which are intended to be used for the alleviation of menopausal symptoms. The level of knowledge on the efficacy and safety of these products differs significantly for each of these products. A wealth of information for example is available for the use of soy isoflavones in menopausal applications forming the basis of an extended scientific opinion of the European Food Safety Authority (EFSA; [5]). Compared to this extensive knowledge base, the available information on other plant products, such as hops, is considerably limited.

According to monograph of the European Medicine Agency hops (strobiles of *Humulus lupulus* L., Cannabaceae) is for the relief of mild symptoms of mental stress and to aid sleep (EMA/HMPC/682384/2013). More recently it was proposed to be used by women for the attenuation of menopausal symptoms [6,7]. Along these lines, we recently observed biological effects of the hops extract regarding ovariectomy-induced deterioration of the bone. In the same study we showed that the hops extract does not induce any unwanted adverse effects in the uterus (uterine wet weight and PCNA protein expression in the luminal uterine epithelia were investigated; [8]).

From the chemically diverse metabolome of *H. lupulus*, four prenylated flavanones and chalcones, namely 6-prenylnaringenin (6-PN), 8-prenylnaringenin (8-PN), isoxanthohumol (IX) and xanthohumol (XH) have most closely been associated with the potential effects of hops and extracts derived thereof [9,10]. XH has been shown to possess chemopreventive properties through induction of detoxifying enzymes [11,12]. It is prone to be metabolized to IX, desmethyl xanthohumol, 8-PN and 6-PN [13,14]. Enhancement of the nontoxic estrogen

2-hydroxylation pathway through AhR mediated up-regulation of the cytochrome P450 enzyme 1A1 has been shown for 6-PN [15]. 8-PN is the major estrogenic constituent in hops extracts [16], described before as 8-isopentenylnaringenin from heartwood of *Anaxagorea luzonensis* A. Gray [17]. On the molecular level it preferentially functions through the estrogen receptor- α (ER α) [16].

Regarding safety of use of a standardized hops extract data from human studies on an intervention study prior to cosmetic surgery showed that 8-PN, the estrogenic constituent of hops, can reach mammary gland after oral consumption of hop extract [18]. However, studies on biological consequences of this observation in human mammary gland tissue are not available, although three smaller trials on hops extracts in the management of menopausal discomfort are available. None of these studies measured mammary gland density, which would be indicative for unwanted side effects in the mammary gland following the application of the extract [6,19,20]. Overall human intervention studies provide mild evidence for clinical efficacy of hops extract in the management of menopausal symptoms, but did not address the issue of breast cancer risk. From animal studies only data on effects on the mammary gland of 8-PN but not of a standardized hops extract are available. The reported 8-PN studies point to a weak estrogenicity and a weak stimulation of proliferative events by this compound within the mammary gland [21-23]. Taken together, these studies showed that 8-PN treatment in higher doses is capable of inducing estrogen-like effects, including regulation of proliferation and of gene expression in the mammary gland.

Whether the estrogenicity contained in the standardized hops extract, due to the presence of 8-PN, is sufficient to drive estrogenic responses in the normal mammary gland or to mimic the tumor promoting activity of E2 in an experimental animal model of breast cancer has never been investigated.

In the current study, we therefore aimed to provide additional information regarding safety of use of a standardized hops extract in animal models for breast cancer risk. In addition to the experimental model of breast cancer, we investigated effects regarding the induction of proliferative events in the normal mammary gland.

2. Material and methods

2.1. Materials

β -Estradiol-3-benzoate (E2-benzoate) was purchased from Sigma-Aldrich (Hamburg, Germany). The isolation and standardization (chemical and biological) of the hops extract has been described previously [24,25]. The extract material was stored at -25°C until use and has shown no detectable signs of degradation over time.

2.2. Ethical approval

Animals used in this study were treated humanely and with regard for the reduction of suffering. The experimental design complied with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines published by the National Centre for the

Replacement, Refinement and Reduction of Animals in Research (<https://www.nc3rs.org.uk/>).

All animal handling and experimental conditions were conducted in accordance to the principles and procedures of the German Federal Law for Animal Welfare and were approved twice, once by the institutional Animal Care and Use Committee of the Technische Universität Dresden and secondly by the state Animal Care and Use Committee. The experiments were finally licensed by the intermediate authority (Landesdirektion Sachsen) with permission numbers 24-9168.11-8/2012-2 and 24-9168.11-1/2013-42.

2.3. Diet

The company Ssniff (Soest, Germany) produced the control and the experimental diets. The experimental diets were based on the R/MH phytoestrogen-free diet. 700 ppm of the hops extract were added for the experiments in mammary gland of Wistar rats and 500 ppm of the hops extract were added for the extended long term tumor experiment in SD-rats. We aimed for an exposure of 56/40 mg/kg BW/d of the hops extract representing an exposure of 0.2/0.14 mg/kg BW/d of 8-PN respectively (based on the assumption of a dietary uptake of 20 g chow by a 250 mg Wistar- or SD- rat). The diet used as positive control in the experiments on normal mammary gland biology of Wistar rats contained 10.5 ppm of E2-benzoate, which has been adjusted to low dose estradiol exposure in comparable experimental settings [26]. As negative control we used the pure R/MH phytoestrogen-free placebo diet.

2.4. Animals

Female Wistar rats (RccHan[®]:WIST; 8-9 weeks of age and 150-200 g) and female Sprague-Dawley rats (Hsd:Sprague Dawley[®] SD[®]; PND 37 and 100-125 g) were obtained from Harlan (Eystrup, Germany). Rats were housed four or five per cage and maintained at 20 ± 1 °C, relative humidity 50-70%, 12 h light – dark cycle and had ad libitum access to the diets and water.

2.5. Experimental procedure – normal mammary gland

The basal setup for the experimental procedures for this part of the experiment are described in connection with the report on the results on bone and uterus from the same study [27] and are schematically depicted in Fig. 1A. In brief, at the time of bilateral OVX, female Wistar rats were divided into feeding groups. The unsubstituted control group (n = 6) received a phytoestrogen-free diet (Ssniff, R/MH, 10 mm, PE-free; Ssniff GmbH, Soest, Germany). The two other groups received phytoestrogen-free diets containing either 10.5 ppm E2-benzoate (n = 6) or 700 mg/kg hops (n = 6) extract respectively. During the intervention period of eight weeks, the animals had free access to water and diet. Eight weeks after the onset of the study, all animals were sacrificed by CO₂-inhalation subsequently to a light O₂/CO₂-inhalation anesthesia. In addition to bone and uteri described elsewhere, mammary glands, which were examined here, were excised, fixed in 4% buffered paraformaldehyde for 24 h and embedded in paraffin. In addition, blood from all animals was collected at the end of the experiment, serum and plasma were prepared and stored at –80 °C until used.

2.6. Experimental procedure – MNU-induced mammary gland carcinogenesis in Sprague-Dawley rats

The experimental procedures for this experimental part are outlined in Fig. 1B. All SD rats were given 50 mg/kg MNU intra-peritoneally on PND 50 and PND 52 in the left and the right side of the abdomen, alternately. Fresh MNU was prepared in 0.9% NaCl adjusted to pH 4.0 with acetic acid. The solubility of MNU in water at room temperature was 1.4% w/v. Animals were randomly allocated to three experimental groups [n = 8 (hops group) or n = 13 [OVX/negative and SHAM/positive controls]. On PND 78, animals were either OVX to reduce the endogenous hormonal background (OVX and OVX + hops group) or underwent a SHAM surgical procedure (SHAM, positive control). Animals in the OVX + hops group received the experimental diet containing the hops extract from PND 79. Animals in the OVX and SHAM groups received the standard control diet. On a weekly basis, body weights and food intake were monitored from PND 44 until PND 219. From these data the average daily exposure was calculated (Suppl. Fig. 1). Tumor presence and number was monitored by palpation from PND 91 to PND 219. The dates of first notice and location of tumors were recorded. Rats were sacrificed when mammary tumors extended more than 2 cm² in size, or if the animals showed an extreme body weight loss or showed another general poor health status.

The experiment was terminated on PND 219. For this, animals were lightly anaesthetized by O₂/CO₂ (1:1) inhalation prior to sacrifice by CO₂ inhalation. Dissected animals were photographed to provide a record of the location of lesions. Tumors and organs were removed, measured and weighed. After excision, tumors were fixed in 10% buffered formalin, embedded in paraffin and processed for histopathological evaluation. In addition, blood from all animal was collected at the end of the experiment. Serum and plasma were prepared and stored at –80 °C until used.

2.7. Histology and histomorphometry

3 mm thick sections were made from embedded mammary glands. Morphological analyses of mammary glands were performed using H&E-stained sections. Immunohistochemical analysis was performed by rehydration and protein epitope retrieval using 10 mM Tris-EDTA (pH 9) and the IHC Select[®] HRP/DAB kit (Merck Millipore, Darmstadt, Germany) according to the manufacturer's protocol. Primary antibodies raised against amphiregulin (AREG; 1:500, Source: rabbit, Bioss Antibodies, Woburn, USA), Ki-67 (1:150, Source: rabbit, Abcam, Cambridge, UK) and progesterone receptor (PR; 1:350, Source: mouse, Thermo Scientific, Rockford, USA) were used. A Keyence BZ-8100E microscope (Keyence, Neu-Isenburg, Germany) was used to visualize tissue sections. Approximately 1000 luminal epithelial cells from all six animals per group were counted to quantify the percentage of AREG-, Ki-67- and PR-positive cells in mammary gland sections.

2.8. Quantitative analysis of flavanones and chalcones in serum and liver

Quantitative analyses of marker compounds in serum were carried out as reported previously [28,29]. Briefly, 0.2 mL of serum was diluted with 0.2 mL of 0.1 M sodium acetate buffer pH 5.0 containing 8-isopentyl naringenin as internal standard and then incubated with 500 units of β -glucuronidase for 3 h at 37 °C to release free aglycones from glucuronic acid

conjugates. Free aglycones were then extracted with 2×2 mL of MTBE. Organic phases were combined, evaporated to dryness and reconstituted in 80% methanol/water (v/v) prior to injection onto LC-MS. LC-MS/MS analyses were carried out on a Shimadzu (Kyoto, Japan) Nexera UHPLC system and LCMS-8050 triple quadrupole mass spectrometer. The analytes were separated on a Waters XBridge 2.0×50 mm $2.5 \mu\text{m}$ C_{18} column using a 3-min gradient from 40 to 70% acetonitrile/0.1% formic acid. The flow rate was 0.4 mL/min and the column temperature was 40 °C. Analytes were quantified using negative ion electrospray and selected reaction monitoring (SRM). Two SRM transitions per analyte were used as described previously. Calibration curves were prepared by spiking standards into blank rat serum and samples processed as described above.

Liver samples weighing between 250 and 360 mg were homogenized in 4 mL of 70% MeOH/water and centrifuged at 15,000g for 15 min. The supernatant was spiked with the internal standard and directly injected onto the column for analysis. The analytes were separated on a Waters XBridge 2.0×50 mm $2.5 \mu\text{m}$ C_{18} column using a 5-min gradient from 24 to 70% acetonitrile/0.1% formic acid. The flow rate was 0.4 mL/min and the column temperature was 40 °C. Analytes were quantified using negative ion electrospray and selected reaction monitoring (SRM) by monitoring both free aglycones and glucuronide metabolites. The quantitative data presented for the liver pertain to free aglycones.

2.9. Statistical analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Bonferroni post-hoc test to determine significant differences compared to the OVX control group. Results were considered statistically significant different at $p < 0.05$.

3. Results

The aim of these studies was to investigate the safety of a standardized hops extract regarding growth of estrogen dependent experimental breast cancer and of proliferative events in the normal mammary gland. We specifically tested a) whether a LC-MS standardized hops extract containing 0.42% of the potent phytoestrogen 8-PN [25] promotes MNU-induced, ER-dependent tumor growth in OVX Sprague-Dawley rats and b) whether it impacts on the proliferative activity in the normal mammary gland following 8 weeks of exposure to the extract.

3.1. Exposure levels

In a previously performed, dose dependent three day uterotrophic assay in OVX rats the doses of 4 mg/kg BW/d, 40 mg/kg BW/d and 400 mg/kg BW/d of the standardized hops extract were tested following treatment by gavage [30]. To enable comparability with the results from this experiment we aimed for an exposure range in our feeding experiment similar to the middle dose in the previous experiment. In the shorter experimental part on the normal mammary gland in Wistar rats 700 ppm of the hops extract resulted in an exposure of around 92 mg/kg BW/d in the first week of the experiment with a decline to around 49 mg/kg BW/d in the last week of the experiment (Table 1). In the longer term tumor experiment, 500 ppm of hops extract were added to the chow resulting in an exposure in the

first 5 weeks between 40 and 45 mg/kg BW/d which leveled around 25 mg/kg BW/d in the last five weeks (Table 1). Over time, we recorded a gradual decline of the exposure level (Suppl. Fig. 1B) due to the increasing body weight (Fig. 2A).

3.2. 8-PN levels in feed, serum and liver

Since 8-PN is the major potent estrogenic compound in the extract data on its relative levels acquired here and elsewhere were set in relation to each other in feed [25], serum (here and [8]) and were in addition compared to relative amounts of XH, IX and 6-PN (Table 1). Applying the feeding pattern described above in 2.5 and 2.6, a remarkable shift of the relative amounts of 8-PN relative to XH, IX and 6-PN became apparent at the end of the experiment. Although 8-PN in the standardized extract is less than 1%, the measured proportions in serum of Wistar- and SD-rats, as wells as in liver of SD-rats was increased relative to the proportion in the chow fed (Table 1). Following a feeding period of 56 days to diet containing 700 ppm of the standardized hops extract we measured 26.8 nM levels of 8-PN in serum of Wistar rats representing 46% of the total flavonoid content (Table 1). After 167 days of feeding a diet containing 500 ppm of the standardized hops extract to Sprague-Dawley rats the 8-PN levels in the serum of these rats were 44 nM representing 68% of the flavonoids. To a certain degree this change of relative levels of 8-PN within the total amount of flavonoids also occurred in the liver. We measured 6.5pg/mg of 8-PN in liver tissue of SD-rats representing 27% of the overall flavonoid content (Table 1). The relative ratios of the other flavonoids within the total amount of flavonoids changed accordingly suggesting a pronounced metabolization of XH, the ratio of which was found to be decreased (Table 1)

3.3. Tumorigenesis experiment with Sprague-Dawley rats – body and tissue weights

In the tumorigenesis experiment, we measured body weight of individual animals (Fig. 2A) and food consumption per cage. From these data we estimated the average food consumption per animal per day as well as the resulting average exposure level as described in 3.1 (see also Supplemental Fig. 1). At each time point following castration, ovary intact animals producing estrogens exhibited a lower body weight than OVX animals. The feeding of the standardized hops extract did not compensate for OVX induced weight gain, which would be indicative of an estrogenic effect regarding this parameter (Fig. 2A).

On the organ level the hops extract neither compensated OVX induced uterine weight loss nor OVX induced reduction of liver weight (Fig. 2B and C). Uterine weight data was further supported because no increased luminal and glandular epithelial height of the endometrium became apparent in response to hops extract (data not shown), which again would have been indicative of an estrogenic effect. Overall, no estrogenicity of the hops extract on body weight gain, uterine weight and uterine epithelial height as well as liver weight was recorded.

3.4. Tumorigenesis experiment with Sprague-Dawley rats – tumor data

Pubertal injection of the carcinogen MNU induces estrogen dependent mammary gland tumors in SD- rats. This MNU induced mammary cancer model in our case was used to assess relative estrogenic potencies of a given compound on estrogen-dependent tumor growth upon estrogen ablation by OVX [31]. We used this approach to test for potential

tumor promoting activities through estrogenic properties associated with the extract. We assessed whether the hops extract can replace estradiol as a tumor promotor if one month of tumor initiation was allowed prior to castration by OVX. All tumor data are summarized in Table 2.

In SHAM operated animals we observed a tumor incidence of 84.6% (11 out of 13 animals) and an average tumor load of three tumors per animal at sacrifice (39 tumors total). The incidence of the tumors in OVX animals was 15.4% (2 out of 13 animals) and the total tumor load at the end of the experiment was 3 tumors. No tumors were detected in the group of animals fed to the hops extract (8 animals). Histopathologically, all tumors which developed in estrogenized animals (SHAM group), had a papillary phenotype, whereas those in OVX animals represented one papillary tumor, one fibroadenoma, and one exhibited a sarcoma-like phenotype. This means two out of three tumors in OVX controls represent tumor entities different to those tumor entities seen under the influence of estrogen in the positive control.

3.5. Proliferative activity in the mammary gland of Wistar rats

The results from the MNU experiment described above provide evidence, that the hops extract is safe regarding estrogenic promotion of mammary gland tumor growth in SD-rats. On the other hand 8-PN, if subcutaneously administered alone at a dose of 15 mg/kg BW/d, stimulated proliferative events in the normal mammary gland of OVX Wistar rats in a three day uterotrophic assay [22]. To contribute to the resolution of potential discrepancies we investigated the effect of eight weeks of oral exposure of castrated female Wistar rats to chow containing 700 ppm of the standardized hops extract on the normal mammary gland. The resulting exposure levels are described in 3.1 and Table 1. At each time point following castration E2-benzoate treated animals exhibited a lower body weight than untreated OVX animals. No compensation for OVX induced weight gain, which would correspond to an estrogenic effect, was detectable for the standardized hops extract (data not shown).

In this second part of the study we primarily focused on potential proliferative events occurring in the normal mammary gland in response to feeding of the hops extract, adding to the tumor growth data described above. Using thin sections and immunohistochemistry, we comparatively assessed the expression of the proliferation markers Ki-67 and AREG, which is induced by binding of estrogen to ER α in the epithelial tissue compartment [32,33] and which binds to epidermal growth factor receptors on the stromal membrane [34]. We also investigated PR as a known example for an estrogen response gene in the normal mammary gland.

After 8 weeks of E2-benzoate treatment we detected a significantly increased percentage of positively stained epithelial cells of the mammary gland for all three marker genes (Fig. 3), if compared to the percentage of labelled epithelial cells in tissue samples from OVX animals. After an experimental period of 8 weeks, treatment with the hops extract unlike treatment with E2-benzoate did not affect the expression of any of the three marker genes (Fig. 3). In other words, 8-PN contained in the extract did not evoke an estrogenic response, which would have been a matter of concern.

4. Discussion

Recently four major mechanisms known to contribute to estrogen-dependent carcinogenesis of the mammary gland and to breast cancer growth were extensively and most comprehensively reviewed regarding the potential modulation by natural compounds, including hops extract and compounds contained therein. These pathways comprise a hormonal, a chemical, an inflammatory, and an epigenetic pathway (Dietz et al., 2016). Our results were derived from an experimental setting, which addressed the potential activation of the estrogen/ER α -dependent hormonal pathway by hops extracts and compounds contained therein. These results provide two pieces of evidence, that a standardized hops extract is not likely to promote growth in the normal or neoplastic mammary gland in an estrogen dependent manner thereby leading to an increased breast cancer risk. This information is important in relation to potential applications of a standardized hops extract for the alleviation of menopausal symptoms. Unlike the ovarian E2 in SHAM operated animals, the extract did not stimulate the growth of MNU-induced mammary gland tumors in castrated SD-rats. In addition, feeding of hops extract did not stimulate the expression of proliferation markers in the normal mammary gland of OVX Wistar rats, which represents an estrogenic response observed following E2-benzoate exposure. This is surprising as relative levels of 8-PN, the major estrogenic compound in the extract, appear to increase in serum and liver if compared to the relative levels of XH, IX and 6-6PN following long term consumption of chow fortified by the extract (Table 1). With the experimental setting described, we mainly focused on investigating potential interactions of hops extract and compounds contained in the extract on the hormonal pathway which is estrogen and ER α -dependent.

In the standardized extract contained in the chow, the 8-PN content represents less than 1% of the total flavonoids. At the end of both experiments, the relative amount of 8-PN in the serum of the experimental animals was found to be increased to a higher relative percentage. 8-PN was found to represent 44% of the overall flavanone and chalcone content in the serum following exposure of Wistar rats to a diet containing 700 ppm of the extract. Following 140 days of exposure to a diet containing 500 ppm of the extract the percentage of 8-PN of the total flavonoid content in the serum reached 68% whereas the liver content in identical animals was 27%. These results suggest that the relative amounts of 8-PN in serum and tissues may accumulate following long term exposure of two different rat strains to a standardized hops extract.

Mechanistically, several processes including the metabolism of XH and IX to 8-PN may contribute to the increase of the relative levels of 8-PN. XH the most prominent flavanone contained in the extract is not only bioavailable in rats, but also metabolized to 8-PN [14,28,35-39]. In addition to biogenesis and enterohepatic recirculation may also contribute to the relatively high levels of 8-PN measured in blood and tissue. In humans results from a pharmacokinetic study are available. The serum concentration-time curves derived from this study were indicative for demethylation of IX to form 8-PN and for cyclization of XN to IX, and therefore indicative of a biotransformation pathway from XN through IX to 8-PN [28].

Another point that needs to be addressed, is the fact that 8-PN, the most potent phytoestrogen known to date [16], behaves weakly estrogenic in vivo if given alone, as shown for uterine [40-42], but also for mammary gland tissue [21-23]. 8-PN delivered as a constituent of an extract or originating from bioconversion of XH and/or IX is only very weakly estrogenic following s.c. application [43], but not at all following oral administration to OVX rodent experimental animals [30,43]. This may primarily be a consequence of the fact that 8-PN exposure from the extract is low (0.2 mg/kg BW/d) as compared to the studies using 8-PN alone (10-15 mg/kg BW/d). At the end of the experiment, levels of 8-PN around 27 nM in the serum of Wistar rats and 46 nM in the serum of SD-rats were measured. At this dose range 8-PN triggers estrogenic responses in MCF-7 breast cancer cells in vitro [22]. Therefore, other mechanisms of protection from estrogenic responses need to be considered.

The hops extract in addition to XH, IX and 8-PN, also contains 6-PN [25], which is represented in the serum and liver tissue at a percentage that corresponds to the relative amount contained in the extract. Whereas 8-PN is the most potent ER α agonist known among the group of phytoestrogenic compounds [16,17], 6-PN has recently been identified as an AhR agonist thereby inducing cytochrome P450 enzyme 1A1 dependent 2-hydroxylation of estradiol, which is believed to be the non-toxic pathway of estrogen metabolism [15]. It is long known that AhR activation e.g. by dioxin in vitro in MCF-7 breast cancer cells leads to functional antagonism of estrogen action [44,45]. This functional antagonism has lately been investigated using 3-methylcholanthrene as an AhR agonist and a genome wide expression analysis by micorarrays as readout. Using this approach we showed that a combinatorial activation of ER and AhR pathways in OVX rats by E₂ (ER agonist) and 3-methylcholanthren (AhR agonist) attenuates the changes of expression of > 70% of the genes triggered in the mammary gland in response to by E₂ [23] and > 80% of the E₂ responsive genes in the uterus [46]. For mammary gland tissue and a selected subset of genes this functional antagonism was also verified for combinatorial versus single treatments of animals with 8-PN instead of E₂ [23]. ER dependent activity of 8-PN may at least in part be functionally counterbalanced by the activation of AhR. As a molecular basis for this hypothesis, on one side we showed that the AhR family of genes is expressed in target tissues in a sex hormone dependent manner [47,48], and on the other side evidence exists that the hops extract may contain AhR activating, counterbalancing compounds with 6-PN [15] as an example.

Overall and in summary, we did not detect indications for any estrogen-dependent growth stimulatory properties of the standardized hops extract in the normal and tumorigenic mammary gland. From these studies, no adverse side effects due to the application of the hop extract in menopausal applications can be delineated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.jsbmb.2017.09.020>.

Abbreviations

AhR	aryl hydrocarbon receptor
AREG	amphiregulin
E2	estradiol
ER	estrogen receptor
HT	hormone therapy
IX	isoxanthohumol
MNU	methylnitroso urea
OVX	ovariectomy or ovariectomized
6-PN	6-prenylnaringenin
8-PN	8-prenylnaringenin
PND	postnatal day
SD rat	Sprague Dawley rat
WHI	women's health initiative
XH	xanthohumol
XRE	xenobiotic response element

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A: Experiment in the normal mammary gland **B: Tumor experiment** **Fig. 1.**

Outline of the experimental approaches. A) *Normal mammary gland* was investigated in castrated (OVX) animals with 56 days of exposure. B) For experiments on *breast cancer growth*, SD rats arrived at the age of PND 37, monitoring of food consumption and body weight started one week later (PND 44), tumor induction with MNU treatment was performed on PND 50 and 52, castration (OVX) was done on PND 78 with animals being allocated to respective diets one day later. Abbreviations: PE-free = phytoestrogen-free, SD rats = Sprague Dawley rats, MNU = Methyl-nitroso-urea, PND = postnatal day.

Fig. 2.

Estrogenicity of the hops extract in OVX SD- rats following tumor initiation with MNU. To follow the course of the tumorigenesis experiment body weight gain was monitored weekly (A). To monitor estrogenicity and potential toxicity of the dietary exposure experiment we measured uterine weight for estrogenicity (B) and liver weight for toxicity (C). Supplementation of the hops extract did not counterbalance effects induced by estrogen ablation.

Fig. 3.

Proliferation and differentiation in normal mammary glands of Wistar rats. The proliferation markers AREG (A) and Ki-67 (B), as well as PR (C) as a marker for differentiation were assessed by immunohistochemistry. Diamonds represent individual animals; boxes indicate 25th to 75th percentiles, and the solid horizontal lines indicate the median, the dotted horizontal lines the mean. The whiskers indicate the highest and the lowest measured values partitioned by the median. Different letters represent significant differences ($p < 0.05$) [one-way analysis of variance (ANOVA)].

Table 1

Relative proportion of 8-PN in the hop extract, serum and liver. The percentage of relative amounts of the four polyphenols 8-PN, XH, IX, and 6-PN in the original extract and in serum and liver samples of experimental animals following two different experimental approaches, comprising two different exposure times are shown. Special emphasis is given on the change of the relative percentage of 8-PN. The summary table shows measured levels and standard deviations of the absolute values, where applicable in relation to minimal and maximal food intake and exposure, which was determined weekly.

Experiment: normal mammary gland in Wistar rats									
Compound	Content in diet ^a (mg/kg)	Total intake (diet) (mg/d)		Exposure (mg/kg/d)		Serum levels ^b (nM)			
		max	min	max	min	max	min	max	min
Extract	700 ^{**}	20.6	18.3	91.7	49.2	–	–	–	–
XN	250.5	7.37	6.6	32.8	17.6	22.6 ± 4.6			
IX	9.5	0.28	0.25	1.2	0.66	5.8 ± 5.6			
8-PN	2.9	0.09	0.08	0.39	0.21	26.7 ± 17			
6-PN	15.3	0.45	0.4	2	1.1	2.9 ± 14			

Experiment: breast cancer in Sprague Dawley rats									
Compound	Content in diet ^a (mg/kg)	Total intake (diet) (mg/d)		Exposure (mg/kg/d)		Serum levels (nM)		Liver levels (pg/mg)	
		max	min	max	min	max	min	max	min
Extract	500 ^{**}	12.1	7.7	45.8	22.3	–	–	–	–
XN	178.9	4.3	2.8	16.4	8	9.4 ± 3.7		8.6 ± 3	
IX	6.8	0.17	0.1	0.62	0.3	7.7 ± 2.5		6.9 ± 2.3	
8-PN	2.1	0.05	0.03	0.19	0.09	43.9 ± 11.4		6.5 ± 2	
6-PN	10.9	0.26	0.17	1	0.49	3.6 ± 1		1.5 ± 0.4	

^a According to the paper by Krause et al. [25].

^b From Keiler et al. [27].

^{**} Estimated amount.

Table 2

Tumor incidence and tumor numbers. To monitor tumor incidence we calculated percentage of tumor bearing animals. To assess tumor take the number of tumors per experimental group was assessed.

Treatment group	Incidence (%; tumor bearing animals/number of animals)	Total number of tumors/group
SHAM (positive control)	85% (11/13)	39
OVX (negative control)	15% (2/13)	3 ^a
Hops (experimental group)	0% (0/8)	0

^aTumors were morphologically different from those developing in the SHAM positive controls.