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Modulation of breast cancer cell survival by aromatase inhibiting hop (*Humulus lupulus* L.) flavonoids

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Abstract

Hop flavonoids are being regarded as attractive molecules to prevent or treat certain forms of cancer. Studies have focused mainly on xanthohumol, the most abundant prenylated chalcone existing in hops extract. However, during the production of beer, or after its ingestion, xanthohumol originates different metabolites, among which isoxanthohumol and 8-prenylnaringenin. The aim of this work was to study the effect of the prenylflavonoids xanthohumol, isoxanthohumol and 8-prenylnaringenin on the breast cancer Sk-Br-3 cell line proliferation, apoptosis and activity of the enzyme aromatase (estrogen synthase). Aromatase activity was determined by a tritiated water assay, cell proliferation was assessed by [^{3}H]thymidine incorporation, sulforhodamine B protein measurement and Ki-67 immunostaining and apoptosis was determined by TUNEL. Our results show that all tested prenylflavonoids were able to inhibit aromatase activity and thus, estrogen formation. Additionally, breast cancer cell line proliferation was decreased and apoptosis induced by all three compounds. The presence of 17β-estradiol in treatment medium was able to revert the effect of the prenylflavonoids on cellular proliferation. These observations strengthen the idea that hop flavonoids may have anti-breast cancer effects and shed new light on a possible mechanism of action by which these effects occur, namely through their ability to decrease estrogen synthesis.

Keywords: Aromatase; Beer; Breast cancer; Estrogens; *Humulus lupulus* L.; Isoxanthohumol; 8-Prenylnaringenin; Sk-Br-3; Xanthohumol

1. Introduction

Breast cancer is the most frequent form of cancer and one of the main responsible for cancer-related mortality in women [1]. Estrogens, female reproductive hormones, play a crucial role in the development of breast cancer, having been shown to be involved in the stages of initiation, promotion and progression of carcinogenesis [2]. Aromatase (EC 1.14.13) is the enzyme responsible for the conversion of circulating androgens into estrogens in the breast. This cytochrome P450 (CYP) isoenzyme (CYP19) is expressed in several tissues where estrogens exert physiological roles [3]. Breast tumors have also been demonstrated to express abnormally high levels of the enzyme, in comparison to normal tissue [4]. In breast tumors, it is most usual that epithelial cells express the lower level of aromatase, the stroma being responsible for the greatest amount of estrogen production [5].

New data show that phytochemicals in common fruits and vegetables may have potential as adjuvants in cancer therapy by their ability to (i) induce apoptosis, (ii) scavenge free radicals (antioxidant activity), (iii) regulate gene expression involved in cell proliferation, cell differentiation, oncogenes and tumor suppressor genes, (iv) modulate enzymes implicated in detoxification (phase II enzymes) and/or the enzymes

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involved in carcinogen activation (phase I enzymes), (v) regulate hormone metabolism, (vi) improve immune system activity and (vii) possess antibacterial or antiviral activities [6–8].

Hop (*Humulus lupulus* L.) extracts are added to beer during its production, being responsible for its flavor and bitterness [9]. These extracts are obtained from the lupulin glands of hop cones and are specially rich in prenylflavonoids [10]. The major prenylflavonoid in hop is xanthohumol (XN), a chalcone which corresponds to 0.1–1% of hop extract in dry weight and accounts for approximately 82–89% of its prenylflavonoids [11]. Lately, much attention has been drawn on this subject, since the discovery that XN bears a wide array of anti-cancer effects. Currently, XN is regarded as a 'broad spectrum' chemopreventive agent because it is able to inhibit initiation, promotion and progression in tumor development; studies on the mechanisms of action of this molecule are on the raising [12].

In previous studies, we have described the ability of compounds in hop and beer to interfere with estrogen synthesis in a choriocarcinoma cell line (JAR cells) [13]. Other important prenylated flavonoids from hops are isoxanthohumol (IXN), which is formed from XN during the brewing process [8] and 8-prenylnaringenin (8-PN) which may be formed non-enzymatically during drying, storage and extraction from hops and enzymatically by demethylation of IXN [14]. These compounds share some of the anti-tumour properties of XN although with differing strength [6,15].

Nevertheless, the mechanisms of action of these polyphenols are largely unknown. Thus, in the present study we aimed at testing the effect of XN, IXN and 8-PN, on aromatase-expressing breast cancer cell line Sk-Br-3 (breast adenocarcinoma-derived) to investigate their putative effects on the modulation of estrogen synthesis. To elucidate the significance of aromatase inhibition on breast cancer cell growth, DNA and protein synthesis, as well as proliferation and apoptosis under the influence of prenylflavonoids were also investigated.

2. Materials and methods

2.1. Materials

[1β-^{3}H]Androst-4-ene-3,17-dione (specific activity 25,3 Ci/mmol, NEN Life Science Products, Boston, MA, EUA). 4-Androstene-3,17-dione, activated charcoal, chloroform, 8-prenylnaringenin, trypsin-EDTA solution, Tris–HCl (Tris–[hydroxymethyl]-aminometane hydrochloride), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (Sigma, St. Louis, MO, USA). Dimethylsulfoxide (DMSO), Triton X-100 (Merck, Darmstadt, Germany). Dextran 70 (Amersham Biosciences, Uppsala, Sweeden). Xanthohumol kindly supplied by Hopsteiner (Mainburg, Germany) through Instituto de Bebidas e Saúde (iBeSa, Portugal). Isoxanthohumol (ALEXIS Biochemicals, Lausen, Switzerland).

2.2. Cell culture

Sk-Br-3 cells were obtained from the American Type Culture Collection. Cells were maintained in humidified atmosphere of 5% CO_2–95% air and were grown in McCoy's 5A culture medium (Gibco BRL, Life Technologies, Gaithersburg, MD, USA) supplemented with 2 mM L-glutamine, 2.2 g/l $NaHCO_3$, 10% heat-inactivated fetal bovine serum (56 °C, 30 min) and 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin. Culture medium was changed every 2–3 days and the culture was split when cells reached confluence. For subculturing, cells were rinsed with PBS and incubated with 0.25% trypsin-EDTA solution (37 °C, 5 min), removed from the plate surface and cultured on 22.1 cm^2 culture plates (Ø60 mm, TPP, Trasadingen, Switzerland).

2.3. Aromatase assay

Aromatase activity was determined as described previously [16], through measuring the release of [^{3}H]H_2O during the aromatization of [^{3}H]androstenedione to estrone. For the experiments cells were split 1:5 and cultured in 12 well plates (3.66 cm^2, Ø22.2 mm, TPP, Trasadingen, Switzerland) for 7 days. To study the effect of compounds, cells were washed with 500 μl serum-free medium and the incubation began with the addition of 300 μl of serum-free medium with 100 nM [^{3}H]androstenedione in the presence of different concentrations of the test compounds or vehicle (1% ethanol). Incubation occurred at 37 °C, in a 5% CO_2–95% air atmosphere for 5 h. After incubation, plates were placed on ice (to stop the reaction) and 250 μl of incubation medium were removed and added to microtubes containing 750 μl of chloroform. Samples were vortexed for 60 s and centrifuged at 9000 × *g*, 1 min. An aliquot of 200 μl of the aqueous upper phase was mixed with the same volume of a 5% charcoal/0.5% dextran 70 suspension, vortexed for 40 s and incubated at room temperature for 10 min. After centrifugation for 15 min at 9000 × *g*, 350 μl of the supernatant were removed to determine the level of radioactivity after addition of 4 ml of scintillation cocktail and the [^{3}H]H_2O was measured by liquid scintillation counting.

2.4. Determination of DNA synthesis

Cells were seeded onto 24-well plates (TPP, Trasadingen, Switzerland) in a final volume of 500 μl culture medium containing 10% FBS. After 24 h in culture, the cells were treated with different concentrations of the prenylflavonoids in the presence or absence of estradiol (10 nM) in culture medium containing 5% FBS for 72 h. Control cells were incubated with 0.1% ethanol. After treatment, the cells were incubated with 200 μl culture medium with methyl-[^{3}H]thymidine (0.5 μCi/well) for 4 h. The medium was removed and the cells were fixed by incubation with 10% trichloroacetic acid (TCA), 1 h at 4 °C. The cells were washed twice with 10%