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Cancer Chemopreventive Potential of Humulones and Isohumulones (Hops α- and Iso-α-acids): Induction of NAD(P)H:Quinone Reductase as a Novel Mechanism

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Phytochemical analysis and chemopreventive testing of a special "α-/β-acid free" hops extract led to the identification of isohumulones (hops iso-α-acids) as potent inducers of NAD(P)H:quinone reductase (QR) activity. CD values (concentrations required to double the specific activity of QR in Hepa1c1c7 cell culture) were in the range of 1.3 to 10.2 µg/mL, with CD value of *trans*-isohumulone < *cis*-isoadhumulone < *cis*-isocohumulone < *cis*-isohumulone (+ *trans*-isoadhumulone). Humulones (hops α-acids) were equally active with CD values of 3.4 to 7.6 µg/mL. However, these activities were accompanied by cytotoxicity. Cohumulinone and humulinone, oxidation products of co- and n-humulone, were inactive. We further identified isohumulones as potent inhibitors of lipopolysaccharide-induced inducible nitric oxide synthase (iNOS) activity in Raw264.7 cell culture, with IC_{50} values of 5.9 – 18.4 µg/mL. Humulones and humulinones were inactive at concentrations < 20 µg/mL. These results indicate that isohumulones, which are considered as the most abundant class of polyphenols in beer, should by further investigated for chemopreventive efficacy in animal models.

Keywords: hops, *Humulus lupulus* L., cancer chemoprevention, NAD(P)H:quinone reductase, hops α-acids, hops iso-α-acids, humulone, isohumulone, humulinone.

Hops (*Humulus lupulus* L.) have been used since ancient times for brewing [1]. It was soon realized that they not only added bitterness and aroma to beer, but also played an important role as a preservative. Subsequently, hops α- and β-acids (humulones and lupulones), constituents of the essential bitter resin, were identified as strong antibiotics against Gram-positive bacteria ([2] and literature cited therein). β-Acids are extremely sensitive to oxidation and do not survive the brewing process. During wort-boiling, the poorly water-soluble humulones are isomerized to isohumulones (iso-α-acids), which are better soluble; this process is involved in the generation of the bitter flavor of beer [3a]. Isohumulones also play an important role in foam stabilization [3b]. Overall, they represent one of the most abundant classes of polyphenols in beer; concentrations of up to 100 mg/L have been reported in very bitter English ales [3c].

Isohumulones are optically active molecules which occur as *cis*- and *trans*-isomers. In analogy to the chemical structures of humulones, three isoforms indicated by the prefix "co-", "-n-" and "ad-" are present in beer, which differ only in their acyl side chain (summary in Fig. 1). Interestingly, Hughes reported that *cis*-isohumulones were more bitter than their *trans*-isomers. In particular, bitterness of the compounds was described as *cis*-isohumulone > *trans*-isohumulone ≈ *cis*-isocohumolone > *trans*-isocohumulone [3b].

In recent years, hops have gained considerable interest because of the biological and potential cancer chemopreventive activities of some of their constituents (reviewed in [4a-c]). As an example, the α-acid n-humulone was described as a potent antioxidant and anti-inflammatory agent capable of inhibiting the induction of cyclooxygenase-2 (Cox-2) in cell culture and mouse skin [5], and displayed

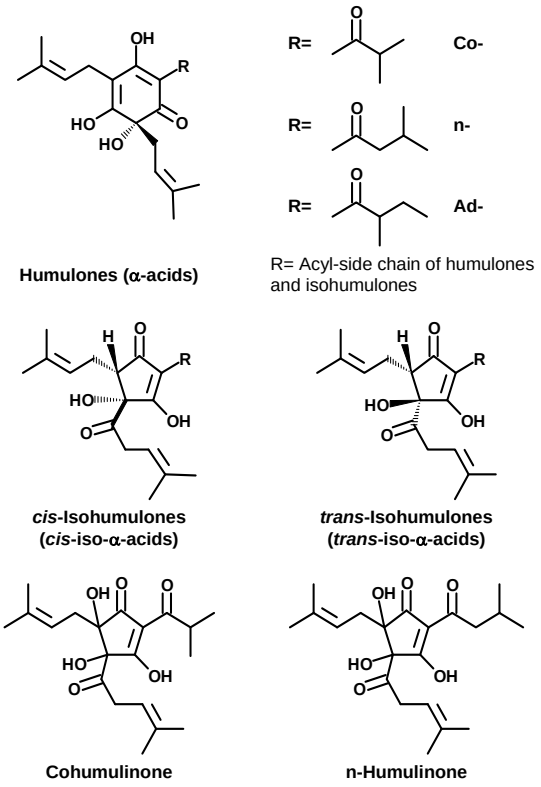

Figure 1: Chemical structures of humulones, *cis*- and *trans*-isohumulones, co- and n-humulinone.

anti-proliferative activity by induction of cell differentiation and apoptosis in HL-60 human promyelocytic leukemia cells *in vitro*. It also inhibited angiogenesis in the chick embryo chorioallantoic membrane (CAM) assay, with a half-maximal effective concentration ED_{50} of 1.5 µg/CAM. Topical application of n-humulone (1 mg) very potently suppressed tumor incidence induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in the two-stage mouse skin model by 93% and tumor multiplicity by 99%. In addition to these cancer preventive effects, humulone was described as a very potent inhibitor of bone resorption and a candidate therapeutic agent for osteoporosis, with a half-maximal inhibitory concentration of 5.9 nM (2.1 ng/mL) in an *in vitro* model of pit formation. Cohumulone was inactive at a concentration of 1 µM (reviewed in [4c]).

Little information is available regarding potential cancer chemopreventive activities of isohumulones. Nozawa et al. demonstrated that freeze-dried beer at a dose of 1%, and isomerized hops extract (IHE) at 0.01 or 0.05% in the diet significantly reduced azoxymethane-induced preneoplastic precursor lesions in rat colon. IHE also potently reduced levels of prostaglandin E_2 (PGE_2) in colonic mucosa, indicating anti-inflammatory potential by inhibition of Cox-2 expression [6a]. Several reports have suggested that isohumulones may have beneficial effects for the treatment of diabetic symptoms by inhibition of aldose reductase and reduction of insulin resistance, hyperlipidemia and obesity by activation of peroxisome proliferator-activated receptor (PPAR) α and γ [6b-f]. They were also shown recently to reduce renal injury in salt-sensitive rats by antioxidant activity [6g].

In continuation of our studies on hops prenylflavonoids [7] and acylphloroglucinol derivatives [8], we here describe results of the phytochemical analysis and chemopreventive testing of a special hops extract which led to the separation of four isohumulones and humulinone, an oxidation product of n-humulone. The chemopreventive potential of these compounds was compared with that of the α-acids cohumulone, n-humulone and adhumulone.

For the isolation of isohumulones we fractionated a commercially available "α-/β-acid free" ethanolic hops extract [9] by size exclusion column chromatography into 18 fractions. Bitter-tasting fraction X08 was separated by semi-preparative HPLC to yield five subfractions X08.A to X08.E. Comparison of NMR and ESI mass spectra with those published [10a-f] led to the following peak assignment: Peak 1 was identified as "n-humulinone", peak 2 as "*cis*-isocohumulone", peak 3 as "*trans*-isohumulone", peak 4 as "*cis*-isohumulone" (maybe plus "*trans*-isoadhumulone), and peak 5 as "*cis*-isoadhumulone". For comparison of potential cancer chemopreventive activities, co-, n-, and adhumulone were isolated from a hops CO_2-extract by counter-current chromatography (modified from [11]). Co- and n-humulinone were synthesized starting from co- and n-humulone according to [10b]. Identities were confirmed by comparison with published spectral data (see Experimental).

Cancer chemoprevention has been defined as the use of chemical agents, natural products or dietary components to block, inhibit, or reverse the development of cancer in normal tissue and preneoplastic lesions [12]. Carcinogenesis is a multi-stage process, which is generally divided into initiation, promotion and progression phases and could be regarded as a continuous accumulation of biochemical and genetic cell damage. The cascade of

events resulting in tumor formation offers a variety of targets for intervention at every stage. Accordingly, fraction X08, its five subfractions X08.A – X08.E containing isohumulones, as well as the purified humulones and humulinones were tested in a series of test systems indicative of cancer chemopreventive potential.

Manifestation of oxidative stress by infections, immune diseases and chronic inflammation has been associated with carcinogenesis in the initiation and promotion phase. Antioxidants may prevent the formation of highly reactive oxidation products, activation of carcinogens, formation of oxidized DNA bases and DNA strand breaks, which have been

Table 1: Summary of potential chemopreventive activities.

Fractions/ Compds	**DPPH**[a]	**QR**		**iNOS**
	SC_{50}	CD	IC_{50}	IC_{50}
X08	>200	2.6	>20	>20
X08.A	132.3	5.0	>20	18.1
X08.B	75.2	7.0	>20	18.4
X08.C	112.5	1.3	>20	5.9
X08.D	74.9	10.2	>20	>20
X08.E	95.5	5.6	>20	9.8
n-Humulinone	>200	>20	>20	>20
Cohumulinone	>200	>20	>20	>20
n-Humulone	5.0	3.4	4.0	>20
Cohumulone	7.2	6.7	9.4	>20
Adhumulone	11.9	7.6	11.5	>20

[a]Test systems: **DPPH**: DPPH scavenging (SC_{50} in µg/mL); **QR**: QR induction (CD, concentration required to double the specific activity of QR in µg/mL, IC_{50} for toxicity in µg/mL); **iNOS**: iNOS inhibition (IC_{50} in µg/mL).

associated with overproduction of reactive oxygen species (ROS) and are involved in the carcinogenic process [13]. We determined radical scavenging potential by reaction with 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals. Consistent with an earlier report [14], the α-acids n-, co- and ad-humulone were identified as potent radical scavengers with half-maximal scavenging concentrations (SC_{50}) of 5.0 µg/mL (13.7 µM), 7.2 µg/mL (20.6 µM) and 11.9 µg/mL (32.9 µM), respectively, as indicated in Table 1. These activities were attributed to the presence of a hydroxyl-group in position C-5 [14] and were only slightly lower than those of ascorbic acid (SC_{50}: 8.5 µM) and the water-soluble Vit. E analog Trolox (SC_{50}: 9.7 µM) [15]. In contrast, fractions containing isohumulones were about 10-fold less potent in scavenging DPPH radicals than the humulones, with SC_{50} values in the range of 75 – 132.3 µg/mL. Oxidation to n-humulinone and cohumulinone further reduced antioxidant activities. Both compounds scavenged DPPH radicals less than 50% at the maximum test concentration of 200 µg/mL.

Xenobiotics, including carcinogens, are metabolized and generally detoxified during phase 1 and 2 metabolism. Phase 2 enzymes, such as glutathione *S*-transferases (GST), conjugate phase 1 metabolites with endogenous ligands and thus enhance their excretion in the form of these conjugates. NAD(P)H:quinone reductase (QR) is not a conjugating enzyme. However, it contributes to detoxification of reactive quinones by 2-electron reduction, thereby preventing the formation of reactive semiquinones and ROS formation by redox cycling [16]. QR activity is induced coordinately with other phase 2 enzymes, making it a well established marker for potential chemopreventive activity [15].

Using QR induction in murine Hepa1c1c7 cell culture as a test system, we identified isohumulones as very potent inducers of QR activity (Table 1). All fractions dose-dependently induced QR activity in a concentration range of 1.25 to 20 µg/mL (Fig. 2). Fraction X08.C containing *trans*-isohumulone was identified as the most active fraction followed by fractions X08.A and X08.E. Humulones also demonstrated good QR-inducing potential with CD values (concentration required to double QR activity) in the range of 3.4 to 7.6 µg/mL. In contrast to isohumulones, these compounds were toxic to the utilized murine hepatoma cells with IC_{50} values of 4.0 to 11.5 µg/mL. The ratio between IC_{50} values and CD values, previously defined as Chemopreventive Index, was close to 1, indicating that these compounds may stimulate their own detoxification [15]. Oxidation of humulones to humulinones completely abrogated QR-inducing potential, but also cytotoxicity. Induction of QR activity by humulones and isohumulones may be explained by activation of the transcription factor Nrf2/Keap-1 pathway similar to other natural products containing "Michael acceptor" functionality [17].

Chronic infections and inflammation lead to nuclear factor κB (NF-κB)-dependent induction of pro-inflammatory enzymes, such as Cox-2 and inducible nitric oxide synthase (iNOS). (Over)production of NO has been linked to early steps in carcinogenesis *via* nitrosative desamination of DNA bases, accumulation of reactive nitrogen oxide species and DNA adduct formation [18]. We and others have shown previously that induction of QR activity is often related to inhibition of iNOS induction [19a-c]. It was, therefore, of interest to analyze whether humulones and isohumulones would inhibit iNOS induction, using the murine macrophage cell line Raw264.7 stimulated with bacterial lipopolysaccharides (LPS) as a model.

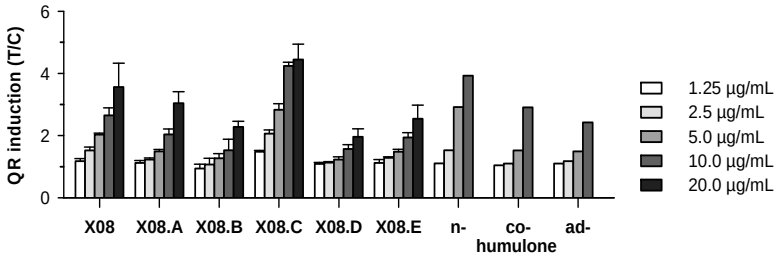

Figure 2: Induction of NAD(P)H:quinone reductase (QR) activity in Hepa1c1c7 cell culture. QR induction was computed by comparison with a solvent treated control (T: treated/ C: control).

In correlation with QR induction, fraction X08.C was most potent in inhibiting LPS-induced iNOS activity with an IC_{50} value of 5.9 µg/mL. These data were in agreement with previous findings of Nozawa et al., who reported that isomerized hops extract and isohumulone inhibited PGE_2 production by Cox-2 in LPS/interferon-γ-stimulated Raw264.7 macrophages [6a]. Neither humulones nor humulinones inhibited iNOS induction in our test system at concentrations up to 20 µg/mL (Table 1). In contrast, TNF-α-mediated Cox-2 expression was potently inhibited by humulone in the murine osteoblastic MC3T3-E1 cell line [20]. Mechanistic investigations indicated that transcription factors NF-κB and NF-IL6 were targets of humulone action. The observed discrepancy of results obtained with humulone in the MC3T3-E1 and Raw264.7 cell lines may be due to differences in the utilized inducers and variations in the signal transduction machinery in both cell lines. Humulone was also reported to inhibit Cox-2 enzymatic activity with an IC_{50} of 1.6 µM [20]. We were not able to reproduce this result at concentrations up to 5 µM using human recombinant Cox-2 as an enzyme source (data not shown, method as described in [7]). In addition to these studies on humulone and isohumulones, Zhao et al. have investigated the potential of other hops constituents, including the β-acid lupulone, xanthohumol, and a series of derivatives of both compounds, to inhibit NO production in the Raw macrophage system [21]. Only chalcones such as xanthohumol were identified as potent inhibitors in this study, whereas lupulone and the β-acid derivatives were inactive.

In conclusion, the present report provides first evidence that induction of phase 2 metabolizing enzymes could contribute to humulone- and isohumulone-mediated cancer chemoprevention. We have identified humulones and isohumulones as novel potent inducers of QR activity. Taking into consideration the relatively high concentrations of isohumulones in beer compared with other bioactive hops components, such as xanthohumol [7], further investigations on potential cancer chemopreventive efficacy and their influence on phase 2 metabolizing enzymes in animal models are warranted. A first indication may be seen in the dose-dependent reduction of carcinogen-induced mammary carcinogenesis by freeze-dried beer [22]. In that study, Nozawa et al. also demonstrated that feeding rats with freeze-dried beer (4% in the diet) increased hepatic GST activity and reduced carcinogen-DNA adducts in mammary tissue. So far, the beer components responsible for these preventive effects have not been analyzed.

Experimental

Plant material: An ethanolic hops extract, as well as a CO_2-hops extract, was produced, as described in [23], from *Humulus lupulus*, var. Taurus (Cannabinaceae) and supplied by Hallertauer Hopfenveredlungsgesellschaft (HHV) mbH, Mainburg, Germany.

General experimental conditions: NMR spectra were recorded on Bruker Avance 500 and Bruker Avance DRX 500 spectrometers in CD_3OD. Mass spectra were measured on a Finnigan MAT 90 mass spectrometer.

Extraction and fractionation: An ethanolic hops extract was treated with supercritical carbon-dioxide to remove hops α- and β- acids. This "α-/β-acid free" fraction is a commercially available hops extract that has recently been introduced into the brewing industry for producing xanthohumol-enriched beers [9]. From this extract, 20 g was separated by size exclusion column chromatography using Sephadex LH-20 (column: Ø 5.5 x 120 cm). A step gradient from methanol/dichlormethane 50:50 (v/v), to 70:30 (v/v) to 90:10 (v/v) was performed to obtain the following fractions X01 (5.80 g), X02 (2.75 g), X03

(2.64 g), X04 (1.41g), X05 (0.39g), X06 (0.67g), X07 (0.95 g), X08 (1.23 g), X09 (0.05 g), X10 (0.64 g), X11 (0.38 g), X12 (0.47 g), X13 (0.18 g), X14 (0.21 g), X15 (0.16 g), X16 (0.06 g), X17 (0.03 g), and X18 (0.01 g).

An intense bitter taste indicated the presence of bitter acids in fractions X07 and X08. Because of higher yield, fraction X08 (330 mg) was further separated by semi-preparative HPLC, which was performed on a RP-18ec column (VP 250/4 Nucleosil 100-5 C18Hop, Macherey–Nagel, Düren, Germany) using acetonitrile/water 56:44 (v/v) with 0.05% TFA. The solvent delivery system was a Waters M-45 (Waters, Milford USA). Peaks were detected with a RI-Detector (RI-Detector 8110, Bischoff, Leonberg, Germany) and, after 7 min, collected to yield 5.1 mg of X08.A, 7.1 mg of X08.B, 5.0 mg of X08.C, 4.6 mg of X08.D and 3.9 mg of X08.E. Structures were determined by NMR spectroscopy (^{1}H, ^{13}C, HSQC, HMBC) and ESI mass spectrometry in comparison with literature data [10b-d,f].

Isolation of humulones as reference compounds: Cohumulone, n-humulone and adhumulone were isolated from a hops CO_2-extract by a modified counter-current separation, as described previously [11]. Identity was confirmed by NMR and mass spectrometry in comparison with literature data [10b, 24a,b].

Synthesis of humulinones: n-Humulinone and cohumulinone were synthesized by partial synthesis, as described in [10b], starting from pure cohumulone and n-humulone, respectively. Structure elucidation was performed as described above. Spectra were in agreement with published literature [10a,b,e].

Determination of potential cancer chemopreventive activities: Experimental details of most test systems utilized in this study are summarized in [7,15]. Briefly, radical scavenging potential was determined photometrically by reaction with 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals in a microplate format [7]. Induction of NAD(P)H:quinone reductase (EC 1.6.99.2) activity in cultured Hepa1c1c7 cells was assayed as described in [25], monitoring the NADPH-dependent menadiol-mediated reduction of MTT [3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide] to a blue formazan. Inhibition of lipopolysaccharide-induced inducible nitric oxide synthase (iNOS) activity (EC 1.14.13.39) in murine Raw 264.7 macrophages was quantified *via* the Griess reaction, as described previously [15,19b].

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