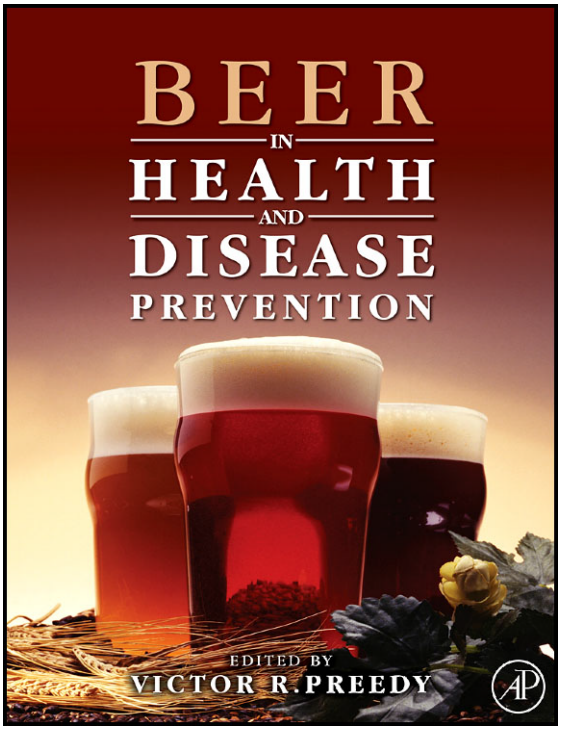

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Phenolic Beer Compounds to Prevent Cancer

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Abstract

Beer is a rich source of dietary polyphenols that are derived from malt (70–80%) and hop (20–30%). Structural classes include simple phenols, benzoic- and cinnamic acid derivatives, coumarins, catechins, di-, tri- and oligomeric proanthocyanidins, (prenylated) chalcones and various flavonoids. We have tested 48 beer phenolics representing 10 different structural classes in a series of test systems indicative of cancer preventive potential. These test systems measured (i) antioxidant effects, for example scavenging of DPPH, superoxide anion, peroxyl and hydroxyl radicals, (ii) modulation of carcinogen metabolism by reducing phase 1 Cyp1A activity and induction of phase 2 NAD(P)H:quinone reductase (QR) activity, (iii) anti-inflammatory mechanisms through inhibition of inducible nitric oxide synthase induction and inhibition of cyclooxygenase 1 activity and (iv) estrogenic and anti-estrogenic effects in Ishikawa cell culture. Gallic acid, catechins, proanthocyanidins and flavonols such as quercetin demonstrated the broadest range of antioxidant activity with scavenging potential against all radicals tested. Quercetin and the hop-derived chalcone xanthohumol were most potent in modulating xenobiotics metabolism. Gallic acid and xanthohumol inhibited iNOS induction, whereas (+)-catechin and (−)-epicatechin were the most potent Cox-1 inhibitors. Overall it became clear that beer compounds belonging to different structural classes have distinct profiles of biological activity in these *in vitro* test systems. Since carcinogenesis is a complex process, combination of compounds with complimentary activities may lead to enhanced preventive effects.

List of Abbreviations

AAPH	2,2,-Azobis-(2-amidinopropane) dihydrochloride
ALP	Alkaline phosphatase
β-PE	β-Phycoerythrin
CD	Concentration required to double the specific enzyme activity
CEC	3-Cyano-7-ethoxycoumarin
CHC	3-Cyano-7-hydroxycoumarin
Cox-1	Cyclooxygenase 1
Cyp1A	Cytochrome P450 1A
DPPH	1,1-Diphenyl-2-picrylhydrazyl radicals
Em	Emission
Ex	Excitation
HHBSS	Hanks balanced salt solution containing 30 mM Hepes, pH 7.8
IC_{50}	Half-maximal inhibitory concentration
iNOS	Inducible nitric oxide synthase
NBT	Nitro blue tetrazolium
$O_2^{-\bullet}$	Superoxide anion radical
OD	Optical density
$OH^{\bullet}$	Hydroxyl radical
ORAC	Oxygen radical absorbance capacity
PMA	Phorbol myristate acetate
PMS	Phenazine methosulfate
QR	NAD(P)H:quinone reductase
$ROO^{\bullet}$	Peroxyl radical
SC_{50}	Half-maximal scavenging concentration
SOD	Superoxide dismutase
XO	Xanthine oxidase

Introduction

Advances in our understanding of the carcinogenic process at the cellular and molecular level made over the past few decades have led to the development of a promising new approach to cancer prevention, termed "chemoprevention" (Sporn and Newton, 1979). Chemoprevention aims to halt or reverse the development and progression of precancerous cells through use of non-cytotoxic nutrients and/or pharmacological agents during the time period between tumor initiation and malignancy (Kelloff *et al.*, 2006). There is a considerable time frame wherein the carcinogenic process could potentially be halted or reversed. Taking this into consideration, the validation and utilization of dietary components, natural products or their synthetic analogs as potential cancer chemopreventive agents in the form of

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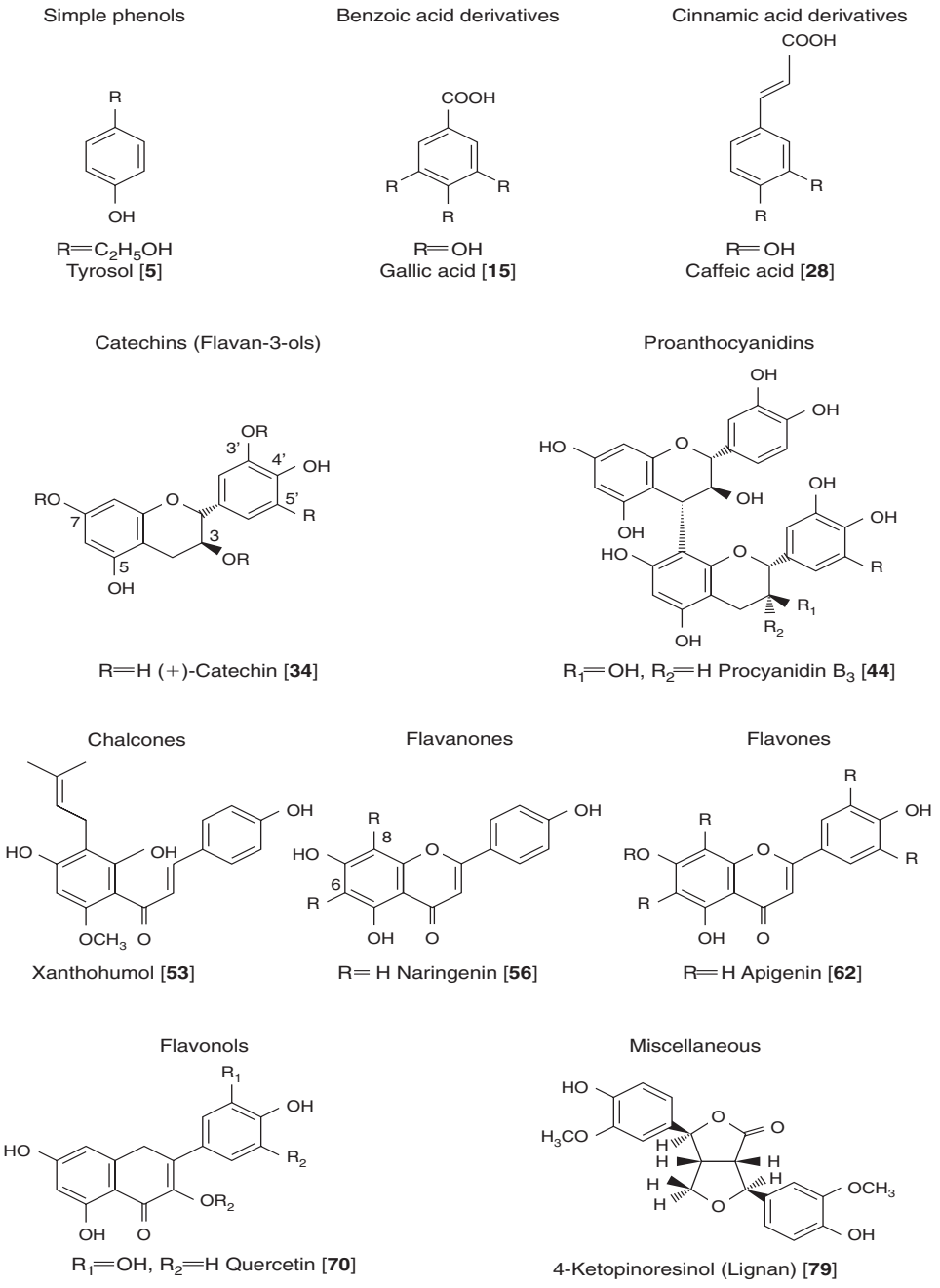

Figure 68.1 Chemical structures of typical phenolic compounds from beer, derived from 10 different structural classes. One typical representative of each class is given as an example.

functional foods or nutraceuticals has become an important issue in current health- and cancer-related research.

There are several lines of evidence suggesting beer as an interesting starting material for chemopreventive agent development. Beer is one of the most consumed beverages worldwide. It is a rich source of nutrient as well as non-nutrient components including vitamins. Beer contains a complex mixture of phenolic constituents, which are derived from malt (70–80%) and hop (20–30%). Structural classes include simple phenols, benzoic- and cinnamic acid derivatives, coumarins, catechins, di-, tri- and oligomeric proanthocyanidins, (prenylated) chalcones and several classes of flavonoids (Figure 68.1).

Polyphenols have been linked to health promoting aspects including the prevention of cancer based on their antioxidant activity and other mechanisms (Yang *et al.*, 2001; Surh, 2003). Recently, several reports have demonstrated that feeding beer or freeze dried beer inhibited chemically induced mammary and colonic carcinogenesis in rats (Nozawa *et al.*, 2004a, b; Nozawa *et al.*, 2006).

Scientific evidence accumulated over the past 10 years points to the cancer preventive potential of selected hop-derived beer constituents including prenylated flavonoids and hop bitter acids (reviewed in Gerhauser, 2005), but a general comparison of beer constituents based on multiple activities relevant for cancer prevention has not been performed so far. To this end, we have investigated the potential cancer chemopreventive activity of 48 beer constituents belonging to 10 different classes of phenolics. Here we summarize the antioxidant activities of these compounds in five different test systems, their influence on the metabolic activation and detoxification of carcinogens, as well as anti-inflammatory properties. Selected hop compounds were also tested for estrogenic and anti-estrogenic properties. Parts of these results have been described previously (Gerhauser *et al.*, 2002a, b; Gerhauser, 2005).

Materials and Methods

Chemicals

All cell culture media and supplements were obtained from Invitrogen (Eggenstein, Germany). Fetal calf serum was provided by PAA Laboratories (Pasching, Austria). β-Phycoerythrin (4 mg/ml), calcein AM, 4-methylumbelliferyl phosphate (MUP), 7-hydroxy-4-methylcoumarin, 3-cyano-7-ethoxycoumarin (CEC) and 3-cyano-7-hydroxycoumarin (CHC) were purchased from Molecular Probes (Mobitec, Göttingen, Germany). The human Ishikawa cell line was obtained from S. Mader, Department of Biochemistry, University of Montreal (Montreal, Canada). All other chemicals were purchased from Sigma Chemical Co. (Deisenhofen, Germany).

Source of phenolic beer compounds

The phytochemical analysis of unstabilized beer and a polyvinylpolypyrrolidone residue obtained during beer stabilization led to the isolation of a total of 51 compounds (Gerhauser *et al.*, 2002b). Tyrosol, salicylaldehyde, vanillin, 4-hydroxyphenylacetic acid, *o*-coumaric acid, *m*-coumaric acid, chlorogenic acid, epicatechin gallate, kaempferol, quercetin, quercitrin, isoquercitrin, rutin and myricetin were also purchased from Sigma Chemical Co. (Deisenhofen, Germany). Syringic aldehyde was obtained from Fluka (Deisenhofen, Germany).

Test Systems to Detect Potential Cancer Chemopreventive Activity

Scavenging of diphenyl-picrylhydrazyl radicals

Radical scavenging potential was determined spectrophotometrically by reaction with 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals in a microplate format at 515 nm (van Amsterdam *et al.*, 1992, with modifications). Briefly, test compounds dissolved in DMSO were treated with a solution of 100 μM DPPH in ethanol for 30 min at 37°C. Scavenging potential was compared with a solvent control (0% radical scavenging) and ascorbic acid (250 μM final concentration, 100% radical scavenging, used as a blank), and the half-maximal scavenging concentration (SC_{50}) was generated from data obtained with 8 serial 2-fold dilutions in a final concentration range of 2–250 μM tested in duplicates. Correction for colored samples was achieved by preparing identical dilutions of the sample in ethanol instead of DPPH solution.

Scavenging of superoxide anion radicals generated non-enzymatically by NADH and phenazine methosulfate

Ewing and Janero (1995) have developed a microassay system suitable for the rapid detection of superoxide anion radical ($O_2^{\bullet -}$) scavengers. Aerobic mixtures of NADH and phenazine methosulfate (PMS) generate $O_2^{\bullet -}$ via the univalent oxidation of reduced PMS. Nitro blue tetrazolium (NBT) can serve as a detector molecule for $O_2^{\bullet -}$ through reduction to a stable formazan product. The reaction mixture (in 0.25 ml total volume) consists of 50 mM phosphate buffer, pH 7.4, containing 0.1 mM EDTA, 50 μM NBT, 78 μM NADH and 3.3 μM PMS (final concentrations). For the assay, 25 μl test sample (stock solution of test compound or superoxide dismutase (SOD) enzyme as a positive control) is pipetted into a microplate well containing 200 μl freshly prepared 0.1 mM EDTA, 62 μM NBT and 98 μM NADH in 50 mM phosphate buffer, pH 7.4. The reaction is initiated by the addition of 25 μl freshly prepared 33 μM PMS in 50 mM phosphate buffer, pH 7.4, containing 0.1 mM EDTA. NBT reduction is continuously monitored at 560 nm over 5 min using a SpectraMax 340PC microplate reader (Molecular Devices, Sunnyvale, CA) in the kinetic mode and with the "automix" function activated. Alternatively, the optical density (OD) at 560 nm can be measured as an endpoint after 5 min of incubation. The percent inhibition of $O_2^{\bullet -}$-dependent NBT reduction by the test sample is calculated, and SC_{50} values are generated from data obtained with 8 serial 2-fold dilutions in a final concentration range of 8–1,000 μM tested in duplicates. SOD (3 U/well) is used as a positive control.

Scavenging of superoxide anion radicals formed enzymatically by xanthine oxidase

Superoxide anion radicals were generated by oxidation of hypoxanthine to uric acid by xanthine oxidase (XO) and quantitated by the concomitant reduction of NBT (Ukeda *et al.*, 1997) adjusted to a 96-well microplate format. The reaction mixture (170 μl), consisting of 50 mM sodium carbonate buffer, pH 9.4, 100 μM EDTA, 25 μM NBT

and 50 μM hypoxanthine, was mixed with test compounds (10 μl, in 100% DMSO, 5% final DMSO concentration) or DMSO (10 μl) as a solvent control. 1 U SOD (in 10 μl 50 mM sodium carbonate buffer, pH 9.4) was used as a positive control. The reaction was started by addition of 12 mU XO (in 10 μl buffer), and the rate of reduction of NBT was monitored for 6 min at 550 nm in a microplate reader (Spectramax 340, Molecular Devices). Alternatively, the yellow tetrazolium salt XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide], which forms a soluble orange formazan dye, was used. Under these conditions, the amount of XO was reduced to 3 mU and measurements were performed at 480 nm. V_{max} values were computed, and the SC_{50} was generated from the data obtained with 8 serial 2-fold dilutions of inhibitors in a final concentration range of 0.8–100 μM tested in duplicates. To exclude a direct inhibitory effect on XO, formation of uric acid was monitored directly at 290 nm under identical conditions as described above without addition of NBT or XTT. In the reaction mixture, 50 μM hypoxanthine was replaced by 100 μM xanthine.

Inhibition of phorbol myristate acetate-induced superoxide anion radical formation in HL-60 cells

Inhibition of phorbol myristate acetate (PMA)-induced superoxide radical formation in HL-60 human promyelocytic leukemia cells differentiated to granulocytes was detected by photometric determination of cytochrome c reduction (Pick and Mizel, 1981; Takeuchi *et al.*, 1994). Briefly, HL-60 cells at a density of 2×10^5 cells/ml were treated with 1.3% DMSO in RPMI 1640 medium containing 100 units/ml penicillin G sodium and 100 units/ml streptomycin sulfate supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 atmosphere for 4 days to induce terminal differentiation to granulocytes. Cells were harvested by centrifugation, washed twice with Hanks balanced salt solution containing 30 mM Hepes, pH 7.8 (HHBSS) and adjusted to a density of 2×10^6/ml. 2×10^5 cells/well (100 μl) were pre-incubated with test compounds (25 μl, in 10% DMSO) for 5 min prior to addition of 75 μl cytochrome c solution in HHBSS (5 mg/ml, 1.25 mg/ml final concentration); 25 μl SOD (600 U/ml in HHBSS, 12 U/well final concentration) was used as a positive control, all other wells obtained 25 μl HHBSS. Superoxide anion radical formation was started by addition of 25 μl PMA (0.55 mg/ml in HHBSS, 55 ng/ml final concentration). The plates were briefly shaken. After an incubation period of 30 min at 37°C, the reaction was stopped by chilling the plates on ice for 15 min. Finally, the plates were centrifuged, and cytochrome c reduction in the supernatant was determined at 550 nm using a microplate reader (Spectramax 340, Molecular Devices). The cell pellet was washed twice with PBS, and cell viability was measured fluorimetrically by enzymatic hydrolysis of the fluorogenic esterase substrate calcein AM (250 nM in PBS, 100 μl/well) at 37°C in a Cytofluor 4000 microplate fluorescence reader (PE Applied Biosystems; excitation 485 nm, emission 620 nm). Using this method, we could avoid unspecific effects of reducing test compounds which falsify commonly used viability assays based on MTT [3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide] or XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] bioreduction. The reaction was linear for at least 30 min. SC_{50} values (half-maximal scavenging concentration of PMA-induced superoxide burst) were generated from the results of 8 serial dilutions of inhibitors tested in duplicate. Only non-toxic inhibitor concentrations resulting in greater than 50% cell viability were considered to calculate scavenging potential.

Measurement of oxygen radical absorbance capacity

For the determination of peroxyl or hydroxyl radical absorbance capacity of test compounds, the oxygen radical absorbance capacity (ORAC) assay (Cao and Prior, 1999) was modified and adapted to a 96-well plate format. β-Phycoerythrin (β-PE) was used as a redox-sensitive fluorescent indicator protein, 2,2,-azobis-(2-amidinopropane) dihydrochloride (AAPH) as a peroxyl radical generator and H_2O_2-$CuSO_4$ as a hydroxyl radical generator. Results were expressed as ORAC units, where 1 ORAC unit equals the net protection of β-PE produced by 1 μM Trolox, a water-soluble Vit. E analog used as a reference compound. Briefly, for the $ORAC_{ROO}$ assay, reaction mixtures contained 170 μl 75 mM sodium potassium phosphate buffer, pH 7.0, 10 μl 9.43 nM β-PE in phosphate buffer and 10 μl of the inhibitor solution (1–5 μM final concentration) or phosphate buffer as a negative control, respectively. The reaction was initiated by addition of 10 μl 320 mM AAPH in phosphate buffer. For the $ORAC_{OH}$ assay, the reaction mixture contained 175 μl 75 mM sodium potassium phosphate buffer, pH 7.0, 10 μl 9.43 nM β-PE in phosphate buffer and 10 μl of the inhibitor solution (1–5 μM final concentration) or phosphate buffer as a negative control. The reaction was initiated by addition of 10 μl of a 1:1 mixture of H_2O_2 (12%) and $CuSO_4$ (0.36 mM in phosphate buffer). The decline of β-PE fluorescence was measured at 37°C until completion for 100 min using a Cytofluor 4000 fluorescent microplate reader (excitation wavelength Ex 530/25 nm, emission wavelength Em 585/30 nm). Results were expressed as ORAC units, where 1 ORAC unit equals the net protection of β-PE produced by 1 μM Trolox. Scavenging capacities > 1 ORAC unit were considered as positive. Current ORAC experiments are performed following the improved protocols by Ou *et al.* (2001, 2002) with fluorescein replacing β-PE as the fluorescent sensor.

Inhibition of Cyp1A activity

β-Naphthoflavone-induced H4IIE rat hepatoma cell preparations were used as a source of cytochrome P450 1A (Cyp1A) enzyme activity. Cyp1A activity was determined *via* the time-dependent dealkylation of CEC to CHC based on the method of Crespi *et al.* (1997, with modifications). Briefly, H4IIE rat hepatoma cells were cultured in 100 mm tissue culture dishes at a density of 5×10^4 cells/ml in MEME medium containing 100 units/ml penicillin G sodium, 100 units/ml streptomycin sulfate and 250 ng/ml amphotericin B supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 atmosphere. After a preincubation period of 24 h, Cyp1A was induced by addition of 10 μM β-naphthoflavone (dissolved in DMSO, 0.1% final concentration). Cells were harvested after 38 h by scraping in 1 ml/plate buffer P (200 mM potassium phosphate buffer, pH 7.4 containing 10 mM $MgCl_2$) and snap frozen in liquid nitrogen. For determination of Cyp1A activity, test compounds dissolved in 10% DMSO (10 μl, final concentration 0.5%) were pipetted to a 96-well microplate. A freshly prepared reaction mixture (100 μl), consisting of 5.2 μl 50 mm $NADP^+$ in water, 4.4 μl 150 mM glucose-6-phosphate in water, 0.5 U glucose-6-phosphate dehydrogenase, 0.1 μl 10 mM CEC (in DMSO) and 90.25 μl buffer P, was then added to each well. The reaction was started by addition of 90 μl cell lysate, passed once through a 27-gauge needle and diluted with buffer P to a final protein concentration of 50–100 μg/ml depending on Cyp1A activity. The rate of dealkylation of CEC to fluorescent CHC was measured for 45 min at 37°C in a Cytofluor 4000 microplate fluorescence reader (PE Applied Biosystems, reading every 2.5 min with prior shaking, excitation 408/20 nm, emission 460/40 nm). V_{max} values were computed, and the half-maximal inhibitory concentration (IC_{50}) was generated from the data obtained with 8 serial 2-fold dilutions of inhibitors in a final concentration range of 0.004–0.5 μM or 0.04–5 μM, respectively, tested in duplicates. Enzyme activities were calculated in comparison with a CHC standard curve and normalized to protein content determined by the BCA method (Smith *et al.*, 1985). α-Naphthoflavone as a known Cpy1A inhibitory compound was used as a positive control.

Determination of NAD(P)H:quinone reductase activity in mouse hepatoma cell culture

For the detection of phase 2 enzyme inducers, quinone reductase (QR) activity was measured in Hepa 1c1c7 cells as described previously (Prochaska and Santamaria, 1988). QR activity was determined by measuring the NADPH-dependent menadiol-mediated reduction of MTT [3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide] to a blue formazan. Protein was determined by crystal violet staining of an identical set of test plates. Induction of QR activity was calculated from the ratio of specific enzyme activities of compound-treated cells in comparison with a solvent control, and CD values (concentration required to double the specific enzyme activity in μM) were generated. In addition, IC_{50} values for the inhibition of cell proliferation were calculated. Only non-toxic inhibitor concentrations resulting in greater than 50% cell viability were considered to calculate inducing potential.

Inhibition of LPS-mediated inducible nitric oxide synthase induction in murine macrophages

Inhibition of lipopolysaccharide (LPS)-mediated inducible nitric oxide synthase (iNOS) induction in murine Raw 246.7 macrophages was determined *via* the Griess reaction (Heiss *et al.*, 2001). Briefly, murine macrophages were cultured in DMEM medium containing 100 units/ml penicillin G sodium, 100 units/ml streptomycin sulfate and 250 ng/ml amphotericin B supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 atmosphere. Cells were plated at a density of $1-2 \times 10^5$ cells/well in DMEM in 96-well plates. After a preincubation period of 24 h, the medium was replaced by 170 μl serum-free DMEM. Inhibitors (10 μl in 10% DMSO, 8 serial 2-fold dilutions, final concentration range 0.8–50 μM) were added, and iNOS was induced by addition of 20 μl LPS solution (500 ng/ml in serum-free DMEM). After 24 h, iNOS activity was determined *via* the quantitation of nitrite levels according to the Griess reaction. Briefly, 100 μl aliquots of cell culture supernatants were incubated with an equal volume of Griess reagent (1% sulphanilamide/0.1% naphthylethylene diamine dihydrochloride/2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 550 nm was determined in a microplate reader and compared to a nitrite standard curve. To determine cytotoxic effects of test compounds, residual cell culture medium was removed, cells were fixed at 4°C for 30 min with 50 μl ice-cold 10% trichloroacetic acid solution in water, washed five times with tap water and briefly dried. Cell numbers were estimated by sulforhodamin B staining (Skehan *et al.*, 1990). Generally, compounds were tested at non-toxic concentrations (cell staining $>$ 50% of LPS-treated control cells).

Inhibition of cyclooxygenase activity

Cox activity was measured at 37°C by monitoring oxygen consumption during conversion of arachidonic acid to prostaglandins in a 1.0 ml incubation cell of an Oxygen Electrode Unit (Hansatech DW, based on a Clark-type O_2 electrode) according to Jang and Pezzuto (1997) with modifications. The reaction mixture, containing 0.1 M sodium potassium phosphate buffer, pH 7.4, 1 mM hydroquinone, 0.01 mM hemin and approximately 0.2 U Cox-1 in 100 μl microsome fraction derived from ram seminal vesicles as a crude source of Cox-1 (specific activity 0.2–1 U/mg protein), was incubated

with 10 µl DMSO (negative control) or inhibitor solution (10 mM in DMSO), respectively, for 90 s. The reaction was started by addition of 2 µl 50 mM arachidonic acid in ethanol (100 µM final concentration), and oxygen consumption was monitored for 20 s. For calculation, the rate of O_2 consumption was compared to a DMSO control (100% activity).

Determination of estrogenic and anti-estrogenic capacity in cultured Ishikawa cells

Measurement of the enhancement of alkaline phosphatase (ALP) activity in the Ishikawa human endometrial adenocarcinoma line allows the assessment of intrinsic estrogenic activity of test compounds. Anti-estrogenic effects are determined by co-treatment with β-estradiol and inhibitors. Cell culture conditions were essentially as described earlier (Littlefield *et al.*, 1990; Markiewicz *et al.*, 1992). Ishikawa cells were routinely maintained in α-MEM medium containing 100 units/ml penicillin G sodium, 100 units/ml streptomycin sulfate and 250 ng/ml amphotericin B supplemented with 10% charcoal-stripped fetal bovine serum at 37°C in a 5% CO_2 atmosphere. One day before the start of an experiment, the medium was changed to an estrogen- and phenol-red-free D-MEM/F-12 mix (1:1) (estrogen-free mix) containing L-glutamate and pyridoxine HCl (Gibco BRL), supplemented with 100 units/ml penicillin G sodium, 100 units/ml streptomycin sulfate, 250 ng/ml amphotericin B and 5% charcoal-stripped fetal bovine serum. For the determination of estrogenic activity, cells were trypsinized with 0.25% phenol-red-free trypsin/EDTA, passed through a 27-gauge injection needle to obtain a single-cell suspension, and plated in 96-well microplates at a density of 2×10^4/well in 200 µl estrogen-free mix. After a preincubation period of 24 h, the medium was replaced by 190 µl fresh estrogen-free mix. Test compounds (10 µl in 10% DMSO, 8 serial 2- or 5-fold dilutions, final concentration range 10^{-12}–10^{-5} M in duplicate) were added to a final volume of 200 µl, and the plates were incubated at 37°C in a humidified 5% CO_2 atmosphere for 72 h. Plates were washed three times with PBS (pre-warmed to 37°C), 50 µl/well 0.5% Triton X in PBS were added, and plates were kept at −80°C overnight. To determine ALP activity, plates were thawed at 37°C for 2 min, 100 µl/well 15 µM MUP in 1 M diethanolamine buffer, pH 9.8, containing 0.24 mM $MgCl_2$ were added, and plates were shaken thoroughly for 5 min on a microplate shaker. Dephosphorylation of MUP to fluorescent 4-methyl-7-hydroxy-coumarin (4-methylumbelliferon) was monitored for 45 min at 37°C (excitation 360 nm, emission 460 nm). ALP activity was determined from the rate of product formation (in arbitrary fluorescent units/min). Protein was determined by SRB staining of an identical set of test plates. For calculation of estrogenic activity, results were expressed as a percentage in comparison with a control sample treated with 50 nM β-estradiol (set as 100%) to determine the half-maximal effective concentration.

For the determination of anti-estrogenic activity, the medium pre-incubated cells (as described above) were changed to 170 µl estrogen-free mix, and cells were treated simultaneously with test samples (10 µl in 10% DMSO) on 10 µl 10% DMSO as a solvent control, respectively, and 20 µl 50 nM β-estradiol in estrogen-free mix. Inhibition of ALP induction was calculated in comparison with the result obtained with the β-estradiol control set as 100%. Tamoxifen was used as a known anti-estrogenic reference compound and produced an inhibition of >50% at a test concentration of 0.5 µM.

Results

We have tested 48 beer constituents for cancer preventive potential in a battery of nine test systems suitable to detect antioxidant and radical scavenging activity (**5.1**), potential to inhibit the metabolic activation of xenobiotics by Cyp1A and to enhance phase 2 detoxification (**5.2**), anti-inflammatory and estrogenic/anti-estrogenic effects (**5.4**).

Antioxidant and radical scavenging capacity

It is well established that excessive production of reactive oxygen species (ROS) caused by immune diseases, chronic inflammation and infections leads to continuous oxidative stress and is causally linked with processes during tumor initiation, promotion and progression (Bartsch and Nair, 2006).

There are three major types of ROS: Hydroxyl radicals ($^{\bullet}OH$) are very reactive oxidants which attack all biomolecular targets (DNA, proteins, lipids …). Superoxide anion radicals ($O_2^{\bullet -}$) do not directly react with biological molecules, but are linked to toxic effects by secondary radical reactions. Other free radicals of the peroxyl type ($ROO^{\bullet}$) contribute by enhancement to oxidative stress (Valko *et al.*, 2006). We have established multiple test systems, including the DPPH radical scavenging assay, three systems to generate superoxide anion radicals (non-enzymatically, enzymatically and cellularly), and the oxygen radical absorbance capacity (ORAC) assay with peroxyl and hydroxyl radicals, to investigate the radical scavenging and antioxidant capacity of phenolic beer compounds. An overview of all tested compounds and their activities in these test systems is given in Table 68.1. To allow a comparison with chemical structures, numbering of the compounds was kept consistent with the Chapter 48. Some examples of phenolic beer constituents are shown in Figure 68.1.

DPPH Radical Scavenging The DPPH assay was included as it is a very simple test system which gives a first indication of radical scavenging potential of a test compound. Due to the bulky structure of the stable DPPH

Table 68.1 Antioxidant and radical scavenging activities of beer constituents

		DPPH scavenging[b]	*Superoxide radical scavenging*			
	Compound[a]	*SC_{50} (μM*[c]*)*	*PMS/NADH SC_{50} (μM)*	*X/XO SC_{50} (μM)*	*$ORAC_{ROO}$ (units)*	*$ORAC_{OH}$ (units)*
	Simple phenols					
5	Tyrosol	>250	>100	>100	2.2	3.8
	Benzoic acid derivatives					
10	*p*-Hydroxy benzoic acid	>250	>1,000	>100	0.9	3.8
13	Protocatechuic acid	**44.8**	**664.6**	**51.9**	**2.8**	**5.6**
15	Gallic acid	**8.5**	**503.7**	>100	**3.1**	3.3
16	Vanillic acid	>250	>1,000	>100	0.5	1.2
17	Syringic acid	44.2	>1,000	>100	1.7	**5.8**
18	Salicylaldehyde	>250	>1,000	n.b.		
20	Vanillin	>250	>1,000	>100	0.3	2.5
21	Syringic acid	>250	>1,000	>100	0.6	3.5
	Phenylacetic acids					
22	4-Hydroxy-phenylacetic acid	>250	>1,000	>100	1.0	4.4
	Cinnamic acids					
24	Cinnamic acid	>250	>1,000	>100	0.1	**8.0**
25	*o*-Coumaric acid	>250	>1,000	>100	0.2	**5.5**
26	*m*-Coumaric acid	>250	>1,000	>100	0.3	3.8
27	*p*-Coumaric acid	>250	>1,000	>100	1.4	**7.9**
28	Caffeic acid	**20.5**	**310.4**	**21.1**	**2.7**	**8.3**
29	Ferulic acid	**47.5**	>1,000	>100	**3.5**	**4.9**
30	Sinapic acid	**31.2**	>1,000	>100	1.0	1.5
31	Chlorogenic acid	**25.6**	n.d.	**75.1**	**2.4**	n.d.
	Flavan-3-ols (catechins)					
34	(+)-Catechin	**16.9**	**116.7**	**23.3**	**4.0**	4.4
35	(−)-Epicatechin	**13.7**	**299.7**	>100	**4.7**	**4.7**
36	Gallocatechin	**15.4**	**52.1**	**11.7**	**2.6**	**7.0**
38	Epicatechin gallate	**12.7**	**116.7**	**23.3**	**7.8**	**4.5**
39	3′-*O*-methylcatechin	**170.6**	>1,000	>100	**4.0**	**5.2**
40	Catechin-7-*O*-β-D-glucopyranosid	**33.1**	**253**	18.2	**2.3**	**4.8**
	Proanthocyanidins					
44	Procyanidin B_3	**7.6**	**55.2**	>100	**5.3**	2.9
49	Procyanidin C	**9.7**	**18.5**	**6.6**	**5.2**	2.5
50	*ent*-Epigallocatechin-(4α→8, 2α→*O*→7)-catechin	**16**	**77.4**	n.b.	**5.2**	2.6
52	2,3-*Cis*-3,4-*trans*-2-[2,3*trans*-3,3′,4′,5,7-pentahydroxyflavan-8-yl]-4-(3,4-dihydroxyphenyl)3,5,7-trihydroxybenzopyran	**11**	>1,000	**14.7**	**3.0**	2.6
	Chalcones					
53	Xanthohumol	>250	>1,000	**31.1**	**2.9**	**8.9**
	Flavanones					
55	Isoxanthohumol	>250	**563.4**	>100	**2.8**	2.1
56	Naringenin	>250	**395.3**	>100	**3.7**	**6.1**
57	8-Prenylnaringenin	>250	**506.7**	>100	1.0	**5.6**
58	6-Prenylnaringenin	>250	**309.4**	**88.5**	1.3	**3.1**
60	5-Methyl-6′-dimethyl-4′,5′-dihydropyrano[2′,3′,7,8]naringenin	>250	>1,000	>100	1.8	**4.3**
	Flavones					
62	Apigenin	>250	>1,000	>100	0.7	**2.7**
64	Tricin	>250	>1,000	70.8	1.9	1.0
65	Apigenin-6-*C*-glucoside (saponaretin)	>250	**157.0**	>100	0.8	1.9
66	Apigenin-6-*C*-glucoside 7-*O*-glucoside (saponarin)	**114.8**	**183.1**	77.6	1.2	1.8
	Flavonols					
68	Kaempferol	**23.7**	**228.8**	**46**	0.9	2.6
70	Quercetin	**8.6**	**67.1**	**18.3**	**5.5**	**4.3**
71	Quercitrin (quercetin-3-*O*-rhamnoside)	**22.4**	n.b.	>100	**2.2**	n.b.
72	Isoquercitrin (quercetin-3-*O*-glucoside)	**15.7**	n.b.	>100	**2.1**	n.b.
73	Rutin (quercetin-3-*O*-rutinoside)	**15**	**65**	**29.7**	**2.6**	**6.1**

Table 68.1 (Continued)

		DPPH scavenging[b]	*Superoxide radical scavenging*			
	Compound[a]	*SC_{50} (μM)*[c]	*PMS/NADH SC_{50} (μM)*	*X/XO SC_{50} (μM)*	*$ORAC_{ROO}$ (units)*	*$ORAC_{OH}$ (units)*
74	Myricetin	**10.4**	**24**	**5.3**	**4.2**	**8.2**
	Miscellaneous compounds					
76	Indole-3-carboxylic acid	>250	>1000	>100	1.7	**4.3**
77	Indole-3-ethanol (tryptophol)	>250	n.d.	>100	n.d.	n.d.
78	1-Methyl-1,3,4,9-tetrahydropyrano [3,4b]indole	>250	>1,000	>100	1.9	3.2
79	4-Ketopinoresinol (lignan)	**191.3**	>1,000	>100	3.7	2.7

Notes: n.d.: not determined; bold print indicates most potent activities.

[a] For chemical structures and further information see Chapter 48. Compounds 1, 2, 3, 4, 6, 7, 8, 9, 11, 12, 14, 19, 23, 32, 33, 37, 41, 42, 43, 45, 46, 47, 48, 51, 54, 59, 61, 63, 67, 69, 75, 80 have not been tested.

[b] The following *in vitro* test systems have been utilized: DPPH: Scavenging of the stable free radical diphenylpicrylhydrazyl; PMS/NADH: Scavenging of superoxide anion radicals generated non-enzymatically by phenazine methosulfate and NADH; X/XO: Scavenging of superoxide anion radicals generated enzymatically during the oxidation of hypoxanthine to uric acid by xanthine oxidase; ORAC: Oxygen radical absorbance capacity assay with peroxyl (ROO) and hydroxyl radicals ($OH^{\bullet}$).

[c] SC_{50}: Halfmaximal scavenging concentration; one ORAC unit equals the net protection of β-PE produced by 1 μM Trolox.

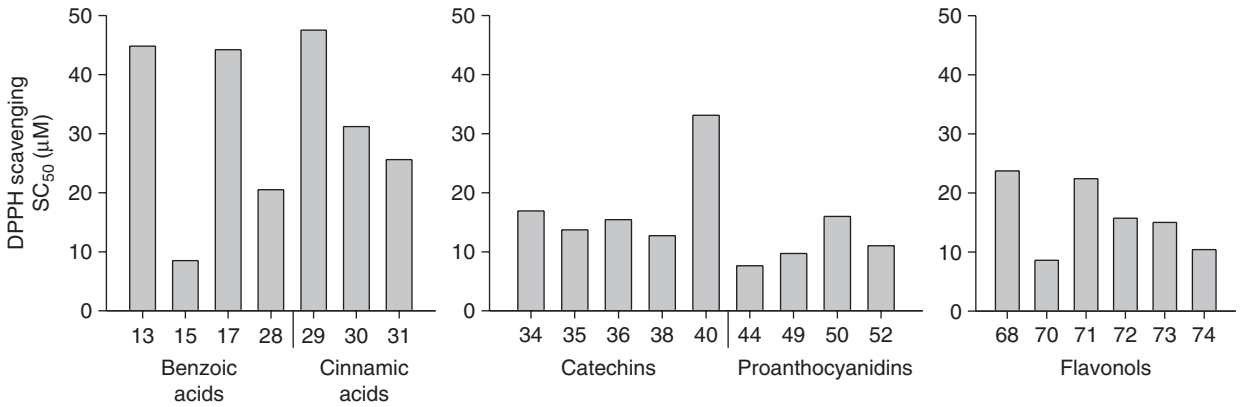

Figure 68.2 DPPH radical scavenging potential of phenolic beer compounds, expressed as the SC_{50}. Only classes with active representatives are shown.

radical, interaction with radical scavengers is sometimes hindered, leading to false negative results.

SC_{50} values of active DPPH scavengers are depicted in Figure 68.2. Overall, it became apparent that the structural classes of catechins (flavan-3-ols), proanthocyanidins and flavonols were most potent in scavenging DPPH radicals.

Gallic acid [**15**] as a benzoic acid derivative with three hydroxyl groups was identified as potent DPPH radical scavenger. The catechins (+)-catechin [**34**], (−)-epicatechin [**35**], gallocatechin [**36**], epicatechin gallate [**38**] and 3′-*O*-methyl-catechin [**39**] were almost equally active in DPPH scavenging with SC_{50} values of 12.7–16.9 μM. A glucosidic linkage at the OH group in position 7 as in [**40**] reduced the SC_{50} in comparison with (+)-catechin [**34**] by about 50%. Procyanidin B_3 [**44**] and prodelphinidin C [**49**] were even more potent than the respective subunits (+)-catechin [**34**] and gallocatechin [**36**], with SC_{50} values of 7.6 and 9.7 μM. Interestingly, neither flavanones such as naringenin [**56**] nor flavones such as apigenin [**62**] were able to scavenge DPPH radicals. In contrast, the flavonols quercetin [**70**] and myricetin [**74**], which have an additional hydroxyl group in position 3 in comparison with flavones, were identified as good scavengers. A glycosidic linkage at the OH group in position 3 as in quercitrin [**71**], isoquercitrin [**72**] and rutin [**73**] reduced the scavenging potential by a factor of 2–3.

Superoxide Anion Radical Scavenging Superoxide anion radicals can be generated chemically, enzymatically or in living cells. We established test systems for all three methods.

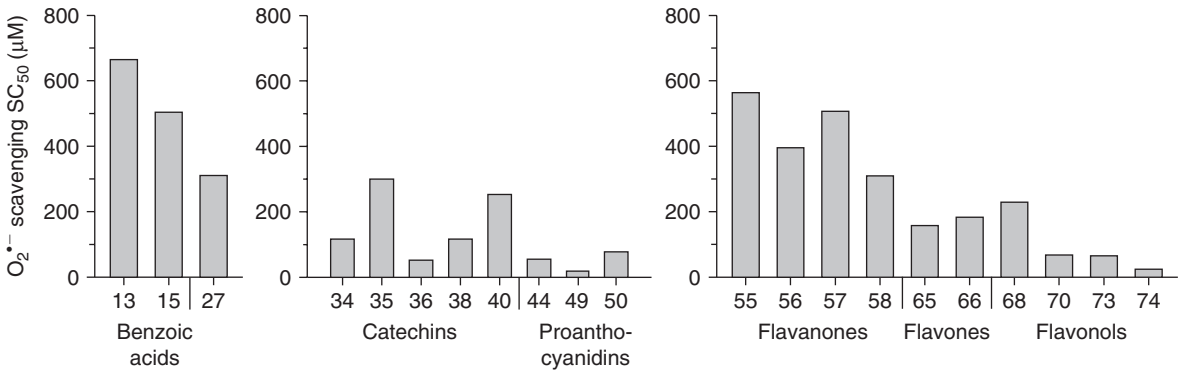

Figure 68.3 Scavenging of superoxide anion radicals generated non-enzymatically in the PMS/NADH system by phenolic beer compounds. The antioxidant potential was expressed as the SC_{50}. Only classes with active representatives are compared.

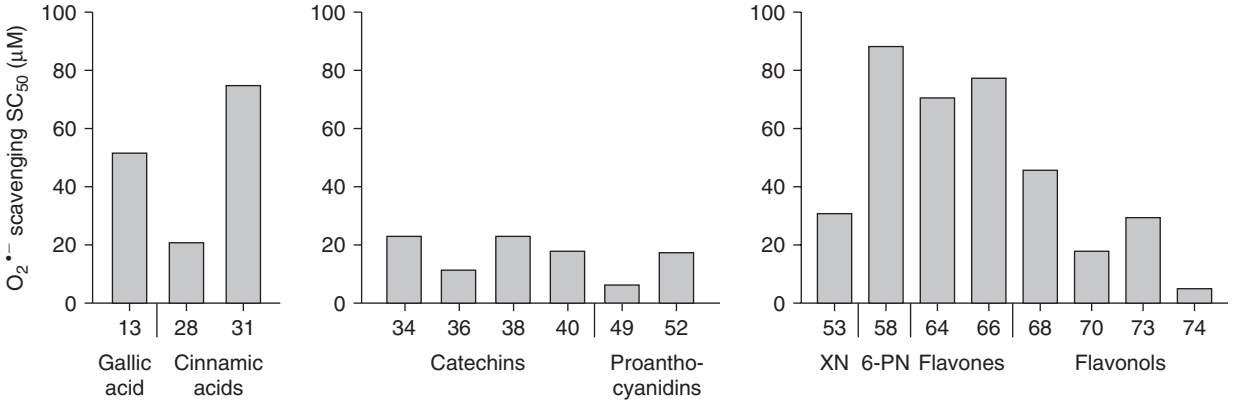

Figure 68.4 Scavenging of superoxide anion radicals generated enzymatically in the xanthine/xanthine oxidase (X/XO) system by phenolic beer compounds. The antioxidant potential was expressed as the SC_{50}. Only results of active compounds are depicted. XN: xanthohumol [**53**]; 6-PN: 6-prenylnaringenin [**58**].

Non-enzymatic PMS/NADH System Non-enzymatically, an aerobic solution of PMS and NADH generates $O_2^{\bullet-}$. The assay was established according to Ewing and Janero (1995). In comparison with the other two $O_2^{\bullet-}$ assays described below, this method apparently generated the highest amount of $O_2^{\bullet-}$, since compounds had to be tested in an about 10-fold higher concentration range up to 1 mM to reach 50% radical scavenging. Results are summarized in Figure 68.3.

In good agreement with DPPH radical scavenging potential, the catechins (+)-catechin [**34**], gallocatechin [**36**] and epicatechin gallate [**38**] potently scavenged superoxide anion radicals, whereas the glucoside [**40**] was less active than the aglycon [**34**]. Again, procyanidin B_3 [**44**] and especially the trimeric proanthocyanidin prodelphinidin C [**49**] were more potent than the respective subunits (+)-catechin [**34**] and gallocatechin [**36**]. The flavanones isoxanthohumol [**55**] as well as naringenin [**56**] and its prenylated derivatives [**57**] and [**58**] showed weak potential to scavenge superoxide anion radicals in this system. Apigenin derivatives saponaretin [**65**] and saponarin [**66**] were more potent scavengers. Overall, the flavonol myricetin [**74**] with three OH groups in positions 3′, 4′ and 5′of the flavonoid structure was identified as the most potent $O_2^{\bullet-}$ scavenger in this assay with a SC_{50} value of 24 μM.

Enzymatic X/XO System Oxidation of hypoxanthine to uric acid by XO leads to the enzymatic generation of superoxide anion radicals. Compounds were tested in a concentration range of up to 100 μM. Most consistently with the previous assays, catechins and proanthocyanidins were identified as the most potent superoxide radical scavengers as shown in Figure 68.4.

(+)-Catechin [**34**], gallocatechin [**36**], epicatechin gallate [**38**] and the glucoside [**40**] all resulted in about 50% scavenging at a concentration of 20 μM. The trimeric prodelphinidin C [**49**] as well as dimer [**52**] were even more potent scavengers, with SC_{50} values of 6.6 and 14.7 μM, respectively,

whereas procyanidin B_3 [**44**] was not able to scavenge superoxide anion radicals efficiently at concentrations below 100 μM. The hop-derived chalcone xanthohumol [**53**] was identified as a superoxide radical scavenger in the X/XO system, but not in the PMS/NADH assay. Consistently with the results obtained in the latter test system, flavanones and flavones were less potent scavengers than flavonols, and myricetin [**74**] was identified as the most potent $O_2^{\bullet -}$ radical scavenger with 50% scavenging of the generated radicals at a concentration of 5.3 μM. Concomitant detection of uric acid proved that the result was not falsified by direct inhibition of XO activity (data not shown).

Cellular System: PMA-Induced Superoxide Burst in Granulocytes Alternatively, superoxide anion radical formation was detected in differentiated HL-60 human promyelocytic leukemia cells stimulated with PMA to generate superoxide anion radicals. Out of 48 compounds tested, only three were more than 50% inhibitory at a test concentration of 100 μM. Dose-dependent inhibition by xanthohumol [**53**], the flavone saponaretin [**65**] and the lignan 5-ketopinoresinol [**79**] is shown in Figure 68.5. These compounds inhibited the PMA-mediated superoxide production with SC_{50} values of 5.5 μM [**53**], 4.7 μM [**65**] and 7.1 μM [**79**]. Interestingly, the lignan 5-ketopinoresinol [**79**] did not show potential to scavenge superoxide anion radicals in the two other assays. This indicated that 5-ketopinoresinol [**79**] may inhibit the signal transduction cascade induced by PMA.

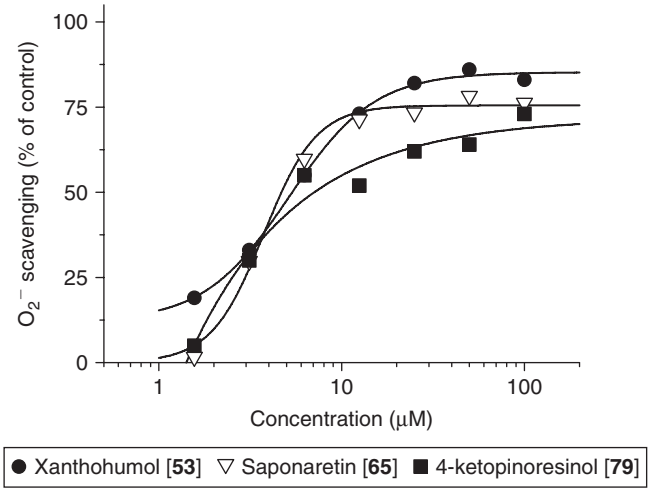

Figure 68.5 Dose-dependent inhibition of phorbol ester-induced superoxide burst in granulocytes by xanthohumol [**53**], saponaretin [**65**] and 4-ketopinoresinol [**79**]. Scavenging of superoxide anion radicals is shown as a percentage in comparison with a solvent control.

Oxygen Radical Absorbance Capacity The ORAC assay is a well-established test system to identify peroxyl and hydroxyl radical scavengers. In the present study, the protocol using β-phycoerythrin (β-PE) as a redox-sensitive fluorescent indicator was adapted to a 96-well plate format. In our current screening assay, we have replaced β-phycoerythrin by fluorescein, due to higher stability of the fluorescence signal and lower price.

$ORAC_{ROO}$ Peroxyl radicals are generated at 37°C by the peroxyl radical generator AAPH. Trolox, a water-soluble Vit. E derivative, is generally used as a reference compound, and one ORAC unit is defined as the net protection of β-PE produced by 1 μM Trolox. For comparison, beer phenolics were also tested at a concentration of 1 μM. 35 out of 48 compounds demonstrated a higher peroxyl radical scavenging potential than Trolox at this concentration. Figure 68.6 summarizes only those compounds which were twice as potent as Trolox (equivalent to at least 2 ORAC units at 1 μM).

The simple phenol tyrosol [**5**], the benzoic acid derivatives protocatechuic acid [**13**] and gallic acid [**15**] as well as caffeic [**28**], ferulic [**29**] and chlorogenic acid [**31**]

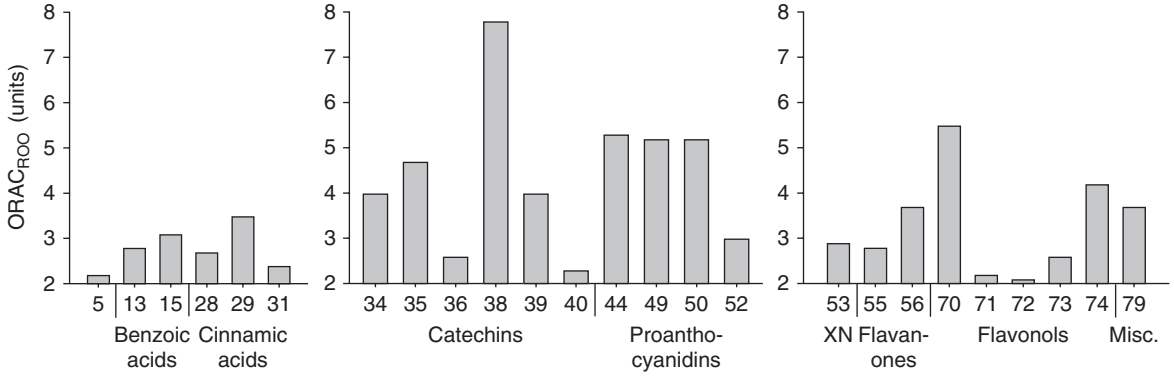

Figure 68.6 Peroxyl radical absorbance capacity ($ORAC_{ROO}$) of phenolic beer compounds. Peroxyl radicals were generated by AAPH and β-phycoerythrin was used as a fluorescent indicator. One ORAC unit is defined as the net protection provided by 1 μM Trolox, a water-soluble Vit. E analog. Compounds were tested at a concentration of 1 μM.

were more potent peroxyl radical scavengers than Trolox. Overall, catechins and proanthocyanidins were the most potent antioxidants in the $ORAC_{ROO}$ assay. Gallocatechin [**36**] was only about half as active as (+)-catechin [**34**] and (−)-epicatechin [**35**], which had a peroxyl scavenging capacity of 4.0 and 4.7 $ORAC_{ROO}$ units, respectively. Esterification of gallic acid [**15**] with (−)-epicatechin [**35**] significantly increased peroxyl scavenging potential, and epicatechin gallate [**38**] was identified as the most potent compound with 7.8 $ORAC_{ROO}$ units. In contrast, blocking of free hydroxyl groups as in [**40**] reduced the scavenging potential in comparison with (+)-catechin [**34**]. Di- and trimeric proanthocyanidins procyanidin B_3 [**44**], prodelphinidin C [**49**] and [**50**] were more potent than the respective subunits (+)-catechin [**34**] and especially gallocatechin [**36**]. Quercetin [**70**], which produced 5.5 ORAC units, was identified as the most potent flavonol.

$ORAC_{OH}$ A combination of H_2O_2 and $CuSO_4$ was used in a Fenton-type reaction to generate hydroxyl radicals. Of notice, the potential of Trolox to scavenge hydroxyl radicals is quite low. Therefore the factor generated from the area between the fluorescence decay curve of the control reaction and that of 1 μM Trolox, which represents 1 $ORAC_{OH}$ unit, was quite low. Consequently, normalization to this factor produced relatively high $ORAC_{OH}$ values for most phenolic beer compounds. In 2002, the authors of the original $ORAC_{OH}$ assay published an improved method termed HORAC, which utilizes fluorescein as a fluorescent sensor, Co(II) fluoride/picolinic acid-H_2O_2 to generate hydroxyl radicals and gallic acid as a reference standard (Ou *et al.*, 2002). In our hands, 1 μM gallic acid was 3.3-fold more potent than 1 μM Trolox. For comparison with gallic acid equivalents (GAE), $ORAC_{OH}$ units summarized in Table 68.1 should be divided by 3.3.

Due to the high number of active compounds, Figure 68.7 shows only those compounds with $ORAC_{OH}$ values above 4. Cinnamic [**24**], caffeic [**27**] and ferulic acid [**28**], gallocatechin [**36**], xanthohumol [**53**] and myricetin [**74**] were identified as the most potent hydroxyl radical scavengers of all beer phenolics investigated.

Modulation of carcinogen metabolism

In addition to the broad spectrum of antioxidant tests, we also investigated the influence of beer compounds to modulate activation and detoxification of xenobiotics. Cyp1A, which is, for example, involved in the activation of carcinogens from cigarette smoke or grilled meat, was used as a model phase 1 enzyme, and NAD(P)H:quinone reductase was tested as a phase 2 enzyme which is induced concomitantly with other phase 2 enzymes such as glutathione *S*-transferases. The results of all compounds which were able to modulate at least one of the activities of these enzymes are summarized in Table 68.2. Compounds which have not been tested in the assays described in Table 68.1 have also not been tested in these systems.

Cyp1A Inhibition Microsomes of β-naphthoflavone-induced rat hepatoma cells were used as a source for Cyp1A activity, and the dealkylation of CEC was monitored over 45 min. As indicated in Figure 68.8a, none of the benzoic and cinnamic acid derivatives [**10–31**], catechins [**34–40**] and proanthocyanidins [**44–52**] inhibited Cyp1A activity more than 50% at the concentration limit of 5 μM. In contrast, xanthohumol [**53**], apigenin [**62**] and quercetin [**70**] were identified as extremely potent Cyp1A inhibitors, with IC_{50} values of 0.02 μM. Glycosides such as saponaretin [**65**] and saponarin [**66**] as well as the quercetin derivatives quercitrin [**71**] and isoquercitrin [**72**] were considerable less active than the respective aglycons. The flavanones isoxanthohumol [**55**], naringenin [**56**] and 8- and 6-prenylnaringenin [**57, 58**] as well as the flavon tricin [**64**] potently blocked Cyp1A activity with IC_{50} values below 0.5 μM. In addition, 1-methyl-1,3,4,9-tetrahydropyrano[3,4-b]indole

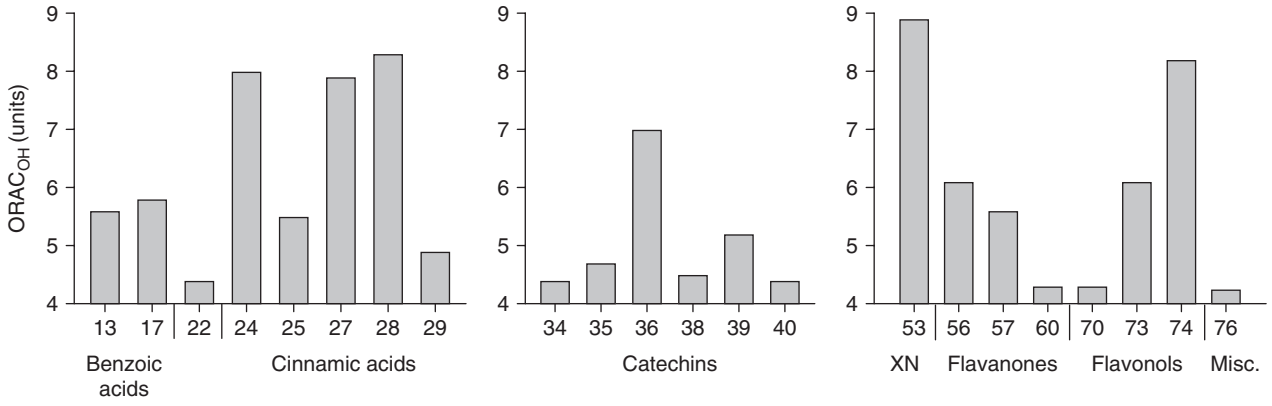

Figure 68.7 Hydroxyl radical absorbance capacity ($ORAC_{OH}$) of phenolic beer compounds. Hydroxyl radicals were generated by $CuSO_4$ and H_2O_2, and β-phycoerythrin was used as a fluorescent indicator. One ORAC unit is defined as the net protection provided by 1 μM Trolox, a water-soluble Vit. E analog. Compounds were tested at a concentration of 1 μM.

Table 68.2 Modulation of xenobiotics metabolism and anti-inflammatory potential of phenolic beer compounds

		Cyp1A inhibition[a]	*QR induction*		*Inhibition of iNOS induction*	*Cox-1 inhibition*
	Verbindung	*IC_{50} (μM)*[b]	*CD (μM)*[c]	*IC_{50}-T (μM)*[d]	*IC_{50} (μM)*	*IC_{50} (μM)*
	Benzoic acid derivatives					
15	Gallic acid	>5	>50	>50	**8.7**	>100 (6)[b]
	Flavan-3-ols (catechins)					
34	(+)-Catechin	>5	>50	>50	>50	**5.2**
35	(−)-Epicatechin	>5	>50	>50	>50	**7.5**
36	Gallocatechin	>5	>50	>50	>50	**45.3**
38	Epicatechin gallate	>5	>50	>50	>50	**26.5**
39	3′-*O*-methylcatechin	>5	>50	>50	>50	**24.8**
	Chalcones					
53	Xanthohumol	**0.02**	**1.7**	7.4	**12.9**	**16.6**
	Flavanones					
55	Isoxanthohumol	**0.3**	**6.5**	29.9	**21.9**	>100 (36)
56	Naringenin	**0.2**	>50	>50	>50	>100 (49)
57	8-Prenylnaringenin	**0.07**	**15.5**	>50	>50	**27.1**
58	6-Prenylnaringenin	**0.09**	**15.4**	>50	>50	>100 (52)
60	5-Methyl-6′-dimethyl-4′,5′-dihydropyrano[2′,3′,7,8]naringenin	**1.6**	**19.6**	>50	>50	>100 (0)
	Flavones					
62	Apigenin	**0.02**	>38.6	38.6	**17.5**	>100 (22)
64	Tricin	**0.13**	>50	>50	>50	>100 (38)
	Flavonols					
68	Kaempferol	>0.5	**5.5**	35.3	**40.6**	>100 (44)
70	Quercetin	**0.02**	2.6	14	**19.8**	>100 (47)
71	Quercitrin (quercetin-3-*O*-rhamnoside)	**3.6**	**5.1**	>50	**29.2**	>100 (15)
72	Isoquercitrin (quercetin-3-*O*-glucoside)	**4.3**	>50	>50	>50	>100 (8)
74	Myricetin	**0.05**	**11.7**	50	>50	>100 (36)
	Miscellaneous compounds					
78	1-Methyl-1,3,4,9-tetrahydropyrano[3,4b]indole	**1.5**	**33.9**	>50	>50	>100 (5)
79	4-Ketopinoresinol (lignan)	**2.2**	**6.0**	34.7	**33**	>100 (15)

Notes: Bold print indicates most potent activities.

[a] Cyp1A: Inhibition of the enzyme activity of cytochrome P450 1A; QR: Induction of the specific activity of NAD(P)H:quinone oxidoreductase (QR) in Hepa1c1c7 murine hepatoma cells; iNOS: Inhibition of lipopolysaccharide-mediated induction of inducible nitric oxide synthase (iNOS) in murine macrophages, measured *via* nitrite levels in cell culture supernatants; Cox-1: Inhibition of sheep-seminal vesicle-derived cyclooxygenase-1 enzymatic activity.

[b] IC_{50}: Halfmaximal inhibitory concentration. Values in parentheses indicate the percentage of inhibition at the indicated concentration.

[c] CD: Concentration required to double the specific activity of QR.

[d] IC_{50}: Concentration that results in 50% inhibition of cell viability.

[**78**] and 4-ketopinoresinol [**79**] displayed moderate Cyp1A inhibitory potential.

NAD(P)H:Quinone Reductase Induction The well-described model of QR induction in cultured Hepa 1c1c7 mouse hepatoma cells was used to characterize the potential of our series of test compound to induce phase 2 xenobiotics metabolism. Antioxidants may induce phase 2 enzymes *via* transcription factor binding to the so-called antioxidant response element. CD values of QR are summarized in Table 68.2 and Figure 68.8b.

The chalcone xanthohumol [**53**] and quercetin [**70**] were identified as most potent inducers of QR activity and we determined CD values of 1.7 and 2.6 μM, respectively. However, both compounds also displayed the highest cytotoxicity with IC_{50}-T values (concentration resulting in 50% inhibition of cell viability), of 7.4 and 14 μM (Table 68.2). Overall, the flavonols kaempferol [**68**], quercetin [**70**] and two quercetin glycosides quercitrin [**71**] and isoquercitrin [**72**] had a higher potential to induce QR activity than the flavanones isoxanthohumol [**55**], 8- and 6-prenylnaringenin [**57, 58**] and the naringenin derivative [**60**]. 1-Methyl-1,3,4,9-tetrahydropyrano[3,4b]indole [**78**] and the lignan 4-ketopinoresinol [**79**] induced QR activity with CD values of 33.9 and 6.0 μM, respectively.

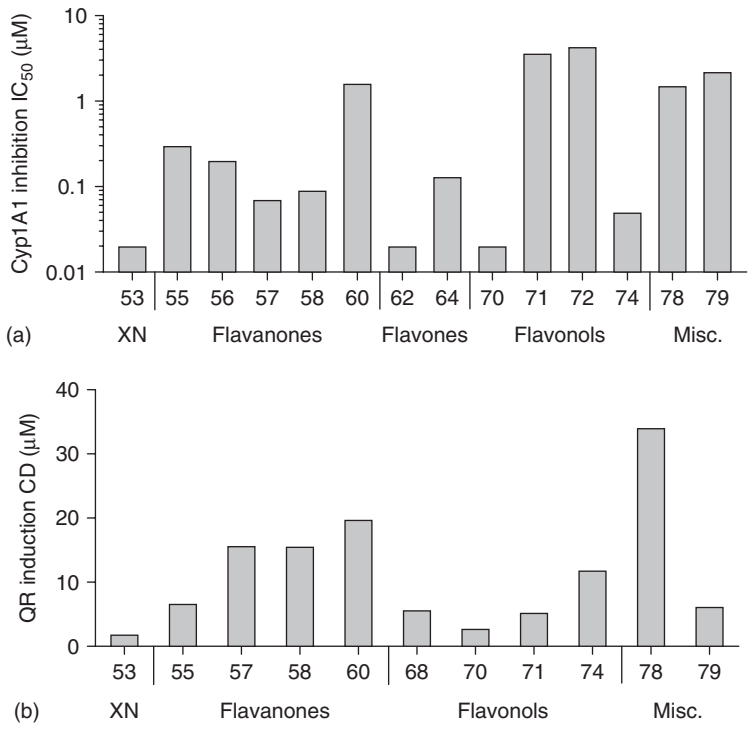

Figure 68.8 Modulation of xenobiotics metabolism. (a) Inhibition of phase 1 Cyp1A activity by beer compounds, measured in β-naphthoflavone-induced H4IIE rat hepatoma cell homogenates by dealkylation of CEC to fluorescent CHC. For comparison of inhibitory potential, IC_{50} are shown of all compounds which resulted in > 50% inhibition at a test concentration of 5 μM. (b) Induction of NAD(P)H: quinone reductase activity as a marker phase 2 enzyme in Hepa 1c1c7 cell culture by beer compounds. Given are CD values of QR in μM.

Anti-inflammatory mechanisms

It is estimated that 18% of all cancer cases are related to chronic inflammatory processes. We therefore tested for potential to inhibit the induction of iNOS and the activity of cyclooxygenase 1 (Cox-1). Both enzymes are involved in the generation of inflammatory mediators in the form of nitric oxide and prostaglandins.

Inhibition of iNOS Induction in Murine Macrophages Inhibition of iNOS induction was measured in LPS-stimulated Raw 264.7 murine macrophage cultures. As summarized in Table 68.2 and Figure 68.9a, only eight of all tested compounds were able to moderately inhibit iNOS induction, that is gallic acid [**15**], xanthohumol [**53**] and its cyclization product isoxanthohumol [**55**], apigenin [**62**], the flavonols kaempferol [**68**], quercetin [**70**] and quercitrin [**71**], as well as 4-ketopinoresinol [**79**], with IC_{50} values in the range of 18.7–40.6 μM. iNOS induction is mediated through activation of the NF-κB signaling pathway (Karin, 2006); therefore, it is likely that these compounds may inhibit iNOS induction by interfering with this pathway (Aggarwal and Shishodia, 2006).

Inhibition of Cox-1 Activity We utilized sheep seminal vesicle microsomes as a source of Cox-1 and measured oxygen consumption during the first step of prostaglandin production as an indication of Cox-1 activity. Notably, only the catechins [**34–39**], xanthohumol [**53**] and 8-prenylnaringenin [**57**] inhibited Cox-1 activity > 50% at concentrations below 100 μM. For (+)-catechin [**34**] and (−)-epicatechin [**35**] we determined IC_{50} values of 5.2 and 7.5 μM. Both compounds were clearly more potent than gallocatechin [**36**], epicatechin gallate [**38**] and 3′-*O*-methylcatechin [**39**]. A sugar linkage at the hydroxyl group in position 7 as in [**40**] completely abrogated Cox-1 inhibitory potential.

Estrogenic and anti-estrogenic activities

Hops have repeatedly been reported to possess estrogenic properties. Therefore, we investigated pro- and anti-estrogenic properties of xanthohumol [**53**] and the (prenylated) flavanones [**55–58**] derived from hops in Ishikawa cell culture. This human endometrial cancer cell line responds to estrogens with elevated ALP activity. Concomitant treatment with estrogens and test compounds allows the identification of anti-estrogens.

Dose-dependent induction of ALP activity is shown in Figure 68.10a. As reported previously (Gerhauser *et al.*, 2002a), xanthohumol [**53**] was identified as a pure anti-estrogen which did not induce ALP activity. In contrast,

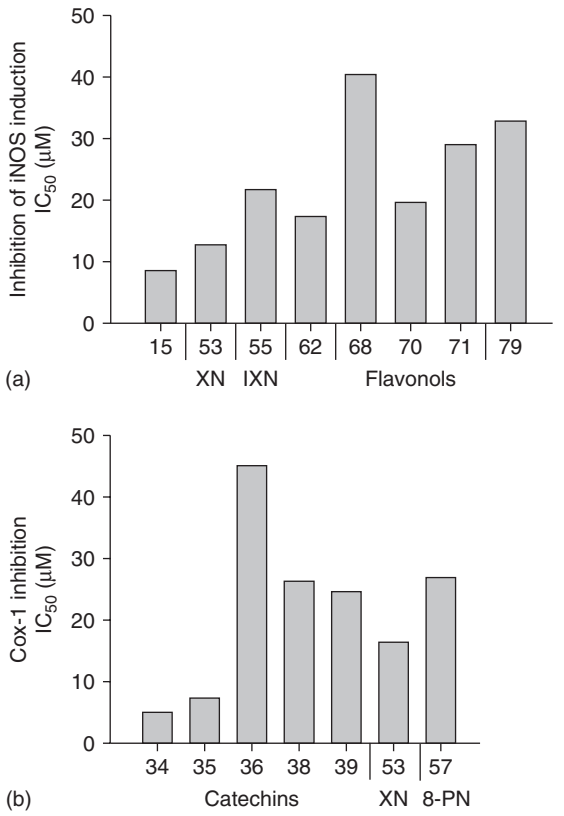

Figure 68.9 Anti-inflammatory mechanisms. (a) Inhibition of LPS-mediated nitric oxide production in Raw 264.7 murine macrophages. IC_{50} are given. XN: xanthohumol [**53**], IXN: isoxanthohumol [**55**]. (b) Inhibition of oxygen consumption during *in vitro* prostaglandin formation by Cox-1. IC_{50} are shown of all compounds which resulted in >50% inhibition at a test concentration of 100 μM. XN: xanthohumol [**53**]; 8-PN: 8-prenylnaringenin [**57**].

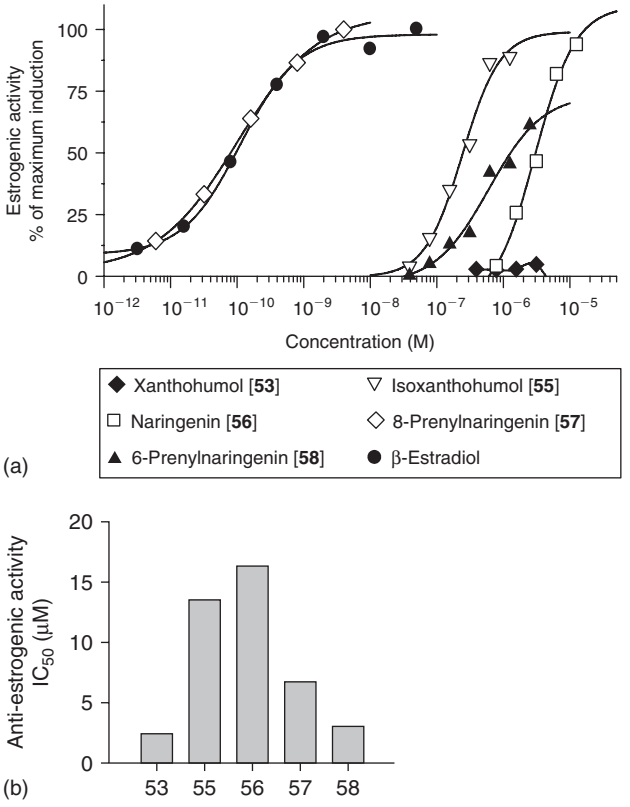

Figure 68.10 Demonstration of estrogenic and anti-estrogenic effects by xanthohumol and selected flavanones. (a) Induction of ALP activity in Ishikawa cell culture as a measure of estrogenic properties. Dose–response curves of xanthohumol [**53**], isoxanthohumol [**55**], naringenin [**56**], 8-prenylnaringenin [**57**], 6-prenylnaringenin [**57**] are shown in comparison with β-estradiol as a percentage of maximum induction. (b) Anti-estrogenic potential of xanthohumol [**53**], isoxanthohumol [**55**], naringenin [**56**], 8-prenylnaringenin [**57**] and 6-prenylnaringenin [**57**]. Ishikawa cells were treated concomitantly with test compounds and 5 nM β-estradiol. Anti-estrogenic activity was determined as inhibition of β-estradiol-induced ALP activity, and IC_{50} values are depicted.

isoxanthohumol [**55**] demonstrated estrogenic potential and induced ALP in a concentration range of 0.1–1 μM. Naringenin [**56**] was about one order of magnitude less active than isoxanthohumol. Prenylation in position C8 strongly increased the estrogenic activity of naringenin by a factor of 10.000, since 8-prenylnaringenin [**57**] was equally estrogenic as β-estradiol used for comparison as a reference compound. This was expected, as 8-prenyl-naringenin [**57**] has been described as one of the most potent phytoestrogens identified so far (Milligan *et al.*, 2002; and references cited in Gerhauser, 2005). On the contrary, prenylation in position C6 as in 6-prenylnaringenin [**58**] only weakly enhanced the estrogenic potential of naringenin [**56**].

All five compounds were also tested for potential anti-estrogenic activity by concomitant application of the compounds in combination with β-estradiol (Figure 68.10b). Xanthohumol [**53**] and 6-prenylnaringenin [**58**] were most effective in inhibiting β-estradiol-mediated induction of ALP activity with IC_{50} values of 2.5 and 3.1 μM. Isoxanthohumol [**55**], naringenin [**56**] and 8-prenylnaringenin [**57**] also inhibited ALP induction, although at higher concentrations. Under *in vivo* conditions the overall response will most likely be an estrogenic effect due to the lower concentrations required for induction of ALP activity in comparison with those required for inhibition of β-estradiol-mediated induction of ALP.

Further Investigations with Xanthohumol

Of all compounds tested, xanthohumol [**53**] was identified as the most promising lead compound for cancer chemoprevention. In addition to the described activities, xanthohumol was tested for anti-proliferative activity. It decreased human recombinant DNA polymerase α activity and inhibited DNA synthesis in MDA MB 435 human breast cancer cells. This led to an arrest of the cell cycle in S-phase (Gerhauser *et al.*, 2002a). Poly(ADP-ribose)polymerase

(PARP) cleavage, activation of caspases-3, -7, -8 and -9 and downregulation of Bcl-2 protein expression were found to contribute to apoptosis induction in cultured human colon cancer cells (Pan *et al.*, 2005). Xanthohumol was also shown to induce cell differentiation in HL-60 human leukemia cells (Gerhauser *et al.*, 2002a) and demonstrated anti-angiogenic potential in a human *in vitro* anti-angiogenesis assay (summarized in Gerhauser, 2005).

Importantly, xanthohumol at nanomolar concentrations prevented carcinogen-induced preneoplastic lesions in mouse mammary gland organ culture (MMOC), providing a first direct indication for its chemopreventive potential (Gerhauser *et al.*, 2002a). Ongoing investigations determine mammary cancer preventive potential of xanthohumol in a long-term carcinogenesis model.

Summary Points

- Beer contains a complex mixture of phenolic compounds belonging to at least 10 different structural classes. In the present study, 48 beer compounds were tested in a series of *in vitro* bioassays indicative of cancer preventive potential. Notably, various structural classes of compounds had quite distinct profiles of activities.
- Overall, catechins and proanthocyanidins were identified as the most potent radical scavengers against DPPH, superoxide anion and peroxyl radicals. Cinnamic acids, xanthohumol and myricetin were most potent in scavenging very reactive hydroxyl radicals.
  With respect to modulation of xenobiotics metabolism, especially xanthohumol and various classes of flavonoids demonstrated potent effects: Xanthohumol, (prenylated) flavanones and the aglycons apigenin, quercetin and myricetin were identified as very potent inhibitors of Cyp1A activity at nanomolar concentrations. On the other hand, xanthohumol, flavanones and flavonols enhanced NAD(P)H:quinone reductase activity as an indication for elevated detoxification.
- Anti-inflammatory activity by inhibition of LPS-induced iNOS activity was provided by gallic acid, xanthohumol, isoxanthohumol, apigenin, selected flavonols and 4-ketopinoresinol, whereas catechins, xanthohumol and 8-prenylnaringenin were identified as Cox-1 inhibitors.
- Based on these results, it is likely that the combination of compounds with different activity profiles might enhance a potential biological effect *in vivo*. This aspect should be paid more attention in future studies.

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