# Cancer Chemopreventive Activity of Xanthohumol, a Natural Product Derived from Hop[1]

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## Abstract

**Characterization and use of effective cancer chemopreventive agents have become important issues in public health-related research. Aiming to identify novel potential chemopreventive agents, we have established an interrelated series of bioassay systems targeting molecular mechanisms relevant for the prevention of tumor development. We report anticarcinogenic properties of Xanthohumol (XN), a prenylated chalcone from hop (*Humulus lupulus* L.) with an exceptional broad spectrum of inhibitory mechanisms at the initiation, promotion, and progression stage of carcinogenesis. Consistent with anti-initiating potential, XN potently modulates the activity of enzymes involved in carcinogen metabolism and detoxification. Moreover, XN is able to scavenge reactive oxygen species, including hydroxyl- and peroxyl radicals, and to inhibit superoxide anion radical and nitric oxide production. As potential antitumor-promoting mechanisms, it demonstrates anti-inflammatory properties by inhibition of cyclooxygenase-1 and cyclooxygenase-2 activity and is antiestrogenic without possessing intrinsic estrogenic potential. Antiproliferative mechanisms of XN to prevent carcinogenesis in the progression phase include inhibition of DNA synthesis and induction of cell cycle arrest in S phase, apoptosis, and cell differentiation. Importantly, XN at nanomolar concentrations prevents carcinogen-induced preneoplastic lesions in mouse mammary gland organ culture. Because XN is easily cyclized to the flavanone isoxanthohumol, activities of both compounds were compared throughout the study. Together, our data provide evidence for the potential application of XN as a novel, readily available chemopreventive agent, and clinical investigations are warranted once efficacy and safety in animal models have been established.**

## Introduction

Cancer is a major disease at a worldwide level, accounting for >7 million deaths/year. Progress made in cancer therapy has not been sufficient to significantly lower annual death rates from most epithelial tumor types, and there is an urgent need for new strategies in cancer control (1). Advances in understanding the carcinogenic process at the cellular and molecular level currently allows a more targeted preventive approach by cancer chemoprevention. It aims to halt or reverse the development and progression of precancerous cells through use of noncytotoxic nutrients and/or pharmacological agents during the time period between tumor initiation and progression to malignancy (2, 3), offering a considerable time frame wherein the anticarcinogenic agents could act. Thus, the identification, mechanistic investigation, validation and utilization of dietary components, natural products, or their synthetic analogous as potential cancer chemopreventive agents has become an important issue in current public health-related research (4, 5), *e.g.,* in the form of functional foods or nutraceuticals. Considering the complexity of cancer causes and development, it will be important to provide a variety of cancer chemopreventive agents with different molecular and cellular targets, acting by multiple mechanisms (6, 7).

For the identification of novel cancer chemopreventive agents, we have set up a broad spectrum of cell- and enzyme-based *in vitro* assays with markers relevant for measuring inhibition of carcinogenesis during the initiation, promotion, and progression stage. These bioassay systems offer fast (within days), sensitive, and cost-effective identification and evaluation of lead compounds for the development of effective chemopreventive agents and the elucidation of their mechanism of action. As a measure to detect anti-initiating properties, we focus on the modulation of carcinogen metabolism, *i.e.,* carcinogen activation by Phase 1 Cyp[3] 1A enzymes and detoxification by the Phase 2 enzyme

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[3] The abbreviations used are: Cyp, cytochrome P450; QR, NAD(P)H: quinone reductase; ROS, reactive oxygen species; E2, 17-β-estradiol; hop, *Humulus lupulus* L; 8-PN, 8-prenylnaringenin; XN, xanthohumol; IX, isoxanthohumol; MMOC, mouse mammary organ culture; FBS, fetal bovine serum; β-PE, β-phycoerythrin; 4-MUP, 4-methylumbelliferyl phosphate; 4-MU, 4-methylumbelliferone; CEC, 3-cyano-7-ethoxycoumarin; CHC, 3-cyano-7-hydroxycoumarin; Cox, cyclooxygenase; NMR, nuclear magnetic resonance; NF, naphthoflavone; NBT, nitroblue tetrazolium; CAT, chloramphenicol acetyltransferase; ORAC, oxygen radical absorbance capacity; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; NO, nitric oxide; LPS, lipopolysaccharide; ALP, alkaline phosphatase; CD, concentration required to double the specific activity of QR; DMBA, 7,12-dimethlybenz[*a*]anthracene; ARE, antioxidant-responsive element; XRE, xenobiotic responsive element; PG, prostaglandin.

QR and on the prevention of oxidative damage by scavenging of ROS and inhibition of NO production. With respect to antitumor promoting activity, we have established models to measure the influence of potential chemopreventive agents on generation or effects of endogenous tumor promoters, *i.e.,* PGs and E2. Finally, for the inhibition of carcinogenesis in the progression phase, we investigate a series of complementary antiproliferative mechanisms, *i.e.,* inhibition of DNA synthesis and cell cycle progression and induction of apoptosis and terminal cell differentiation.

Hop is an important source of phenolic compounds in beer. The dried hop cones contain 4–14% polyphenols, mainly phenolic acids, chalcones, flavonoids, catechins, and proanthocyanidins. In addition, hop provides a resin consisting of bitter compounds such as humulones and lupulones (8, 9). Hop cones are used therapeutically as mild sedatives and have been listed in the European Pharmacopoeia (10). There are several lines of evidence suggesting beer and hop as promising starting materials for chemopreventive agent development. In 1999, beer was reported to possess antimutagenic effects and was shown to inhibit DNA-adduct formation (11, 12), and betaine glycine was identified as an active principle against 2-chloro-4-methylthiobutanoic acid, the sanma-fish mutagen (13). Recently, prenylated flavonoids from hop were shown to modulate drug metabolism *in vitro* by inhibition of various Cyp enzymes and by induction of QR activity in murine hepatoma cells (14, 15). In addition, antioxidant (16) and cytotoxic effects have been described previously (17), and 8-PN isolated from hop has been identified as a potent phytoestrogen (18).

To this end, we have investigated the potential cancer chemopreventive activity of constituents in beer and beer raw materials in our bioassay systems. In this report, we summarize anticarcinogenic properties of XN, a prenylated chalcone from hop, and its cyclization product IX at the initiation, promotion, and progression stage. We demonstrate that XN prevents preneoplastic lesion development in MMOC at nanomolar concentrations, and we conclude that this high efficacy might be because of the combination of multiple inhibitory mechanisms, resulting in an additive or synergistic amplification of chemopreventive activity.

## Materials and Methods

**Chemicals.** All cell culture material was obtained from Life Technologies, Inc. (Eggenstein, Germany). FBS was from Greiner Labortechnik GmbH, (Frickenhausen, Germany). dTTP (60–80 Ci/mmol, 1 mCi/ml in 50% ethanol) was purchased from Biotrend (Cologne, Germany). $\beta$-PE (4 mg/ml), calcein AM, 4-MUP, 4-MU, CEC, and CHC were purchased from Molecular Probes (Mobitec, Göttingen, Germany). Human DNA polymerase $\alpha$ was a gift of Heinz-Peter Nasheuer (Institute of Molecular Biotechnology, Jena, Germany). Human recombinant Cox-2 was provided by Denis Riendeau (Merck Frosst Centre for Therapeutic Research, Kirkland, Quebec, Canada). The human Ishikawa cell line was obtained from Sylvie Mader (University of Montreal, Montreal, Quebec, Canada). All other chemicals were purchased from Sigma Chemical Co. (Deisenhofen, Germany).

**Isolation of XN and IX from Hop.** XN and IX were isolated from a commercially available ethanolic hop extract (Hallertauer Northern Brewer) obtained from Karlsberg Brauerei (Homburg/Saar, Germany). The extract (30 g) was dissolved in methanol/$CH_2Cl_2$ (1:1 v/v) and chromatographed on Sephadex LH 20 with methanol/$CH_2Cl_2$ (1:1 v/v) and (4:1 v/v) eluent. Fractions of 15 ml were collected and monitored by silica gel thin layer chromatography. The fractions containing flavonoids were combined, evaporated, and subjected to bioassay testing. Active fractions were separated on silica gel via vacuum liquid chromatography with a hexane/EtOAc gradient to yield XN (168.3 mg) and IX (8.0 mg) and a series of related compounds. For $^1$H NMR of XN (in $CDCl_3$), $\delta$: 1.6 (s, 3H); 1.69 (s, 3H); 3.13 (d, $J = 7.1$ Hz, 2H); 3.85 (s, 3H); 5.13 (t, $J = 7.1$ Hz); 6.06 (s), 6.83 (d, $J = 8.6$ Hz); 6.83 (d, $J = 8.6$ Hz); 7.55 (d, $J = 8.6$ Hz); 7.55 (d, $J = 8.6$ Hz); 7.65 (d, $J = 15.5$ Hz); and 7.75 (d, $J = 15.5$ Hz); (Bruker DRX 500-Spectrometer). For $^{13}$C NMR of XN ($CDCl_3$), $\delta$: 17.5, 20.9, 25.3, 55.6, 91.0, 104.5, 107.3, 115.9, 115.9, 123.0, 123.8, 126.0, 129.7, 130.3, 130.3, 142.3, 159.8, 160.4, 162.4, 164.5, and 191.5 (Bruker DRX 500-Spectrometer). Formula: $C_{21}H_{22}O_5$. Analysis: calculated C 71.2, H 6.3, O 22.6; Found: C 70.40, H 6.75, O 22.85. For $^1$H NMR of IX (in $CDCl_3$), $\delta$: 1.55 (s, 3H); 1.61 (s, 3H); 2.66 (dd, $J = 16.9/2.9$ Hz); 2.97 (dd, $J = 17.0/12.4$ Hz); 3.19 (d, $J = 6.9$ Hz, 2H); 3.79 (s, 3H); 5.13 (t, $J = 7.3$ Hz); 5.28 (dd, $J = 12.3/2.9$ Hz); 6.11 (s); 6.80 (d, $J = 8.7$ Hz); 6.80 (d, $J = 8.7$ Hz); 7.30 (d, $J = 8.7$ Hz); and 7.30 (d, $J = 8.7$ Hz). For $^{13}$C NMR of IX ($CDCl_3$), $\delta$: 17.9, 22.5, 25.5, 46.3, 55.9, 79.5, 93.8, 93.8, 106.2, 116.1, 116.1, 123.9, 128.7, 128.7, 131.0, 131.0, 158.2, 158.2, 161.0, 161.0, and 192.8. Formula: $C_{21}H_{22}O_5$. Analysis: calculated C 71.2, H 6.3, O, 22.6; Found: C 70.97, H 5.89, O 23.14. $^1$H- and $^{13}$C-NMR spectral data were in agreement with those reported in the literature (19, 20).

**Anti-initiating Mechanisms: Modulation of Carcinogen Metabolism.** Homogenates of H4IIE rat hepatoma cells induced for 39 h with the Cyp1A inducer $\beta$-NF at a concentration of 10 $\mu$M were used as an enzyme source to measure Cyp1A activity. The rate of time-dependent dealkylation of CEC to CHC was determined fluorimetrically in 96-well plates for 40 min at 37°C using a Cytofluor 4000 fluorescence reader (excitation 408/20 nm, emission 460/40 nm; PE Applied Biosystems; Ref. 21, modified from Ref. 22). CEC has high selectivity for human and rat Cyp1A forms (23), and use of $\beta$-NF-induced cell homogenates additionally enhanced the specificity of the assay for Cyp1A activity. Inhibition constants were generated from Lineweaver-Burk, Dixon, and Cornish-Bowden plots of the results of kinetic experiments (24, 25) with 2.5, 5, and 10 $\mu$M CEC, respectively, as a substrate. The $IC_{50}$ of $\alpha$-NF, a known Cyp1A inhibitor used as a positive control, was 0.005 $\pm$ 0.001 $\mu$M ($n = 4$).

For the detection of Phase 2 enzyme inducers, QR activity was measured in cultured Hepa 1c1c7 murine hepatoma cells ($1.5 \times 10^4$ cells/ml) after a 48-h induction period by the NADPH-dependent menadiol-mediated reduction of 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide to a blue formazan as described previously (21, 26). 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 were generated. β-NF was used as a positive control with a CD value of 0.07 ± 0.02 μM ($n = 4$).

The mechanism of QR induction was analyzed in transient transfection experiments. Hepa 1c1c7 cells were transfected with three different plasmid constructs containing various portions of the 5′ regulatory region of the rat *QR* gene linked to the *CAT* structural gene using the calcium phosphate precipitation method as described previously (26). β-Galactosidase (pCH110; Pharmacia, Piscataway, NJ) was cotransfected. After recovery over night, transfected cells were treated with 2 μM β-NF, 5 μM sulforaphane, or 5 μM XN, respectively (all dissolved in 5 μl of DMSO, 0.1% final concentration), or 0.1% DMSO as solvent control, for 24 h, harvested and lysed. CAT expression in cell lysates was determined using a CAT ELISA kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. CAT activities were normalized for protein content and for β-galactosidase activity, measured by standard methods, and the ratio between compound-treated and control samples was calculated. All data are expressed as mean ± SD. Means were compared using the Student's *t* test with $n = 3$.

To confirm potential to induce Phase 1 enzymes, Cyp1A induction was determined in Hepa 1c1c7 cells grown in 96-well plates at a density of $3 \times 10^4$ cells/ml in 200 μl of α-MEM and treated with test compounds as described previously (21, 26). After an incubation period of 24 h, plates were rinsed three times with prewarmed PBS (pH 7.4), and Cyp1A activity, using 5 μM CEC as a substrate, was measured as described above. Enzyme activities were normalized to protein content determined by crystal violet staining of an identical set of test plates. Cyp1A induction was calculated from the ratio of specific enzyme activities of compound-treated cells in comparison with a solvent control. Treatment of Hepa 1c1c7 cells with 0.5 μM β-NF for 24 h resulted in >35-fold induction of Cyp1A activity.

**Antioxidant Capacity.** Peroxyl- and hydroxyl radical scavenging capacity was analyzed in the ORAC assay (27), modified and adapted to a 96-well plate format (21). β-PE was used as a redox-sensitive fluorescent indicator protein, 2,2,-azobis-(2-amidinopropane) dihydrochloride 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 vitamin E analogue.

Superoxide anion radicals were generated by oxidation of hypoxanthine to uric acid by xanthine oxidase and quantified by the concomitant reduction of NBT according to Ref. 28 adjusted to a 96-well microplate format (21). $V_{max}$s were computed, and the half-maximal scavenging concentration ($SC_{50}$) was generated from the data obtained with five serial 2-fold dilutions of inhibitors in a final concentration range of 6.25–100 μM tested in duplicates. To exclude a direct inhibitory effect on xanthine oxidase, formation of uric acid was monitored directly at 290 nm under identical conditions as described above without addition of NBT. In the reaction mixture, hypoxanthine was replaced by xanthine. Alternatively, superoxide anion radical formation was detected in differentiated HL-60 human promyelocytic leukemia cells by photometric determination of cytochrome *c* reduction (Ref. 21, modified from Ref. 29). Cultured HL-60 cells were treated with 1.3% DMSO to induce granulocyte differentiation. After 4 days, cells were harvested by centrifugation and washed twice with HBSS (pH 7.8) containing 30 mM HEPES (HHBSS). A total of $2 \times 10^5$ cells/well (100 μl) was preincubated with test compounds (25 μl, in 10% DMSO) for 5 min before addition of 75 μl of cytochrome *c* solution in HHBSS (5 mg/ml, 1.25 mg/ml final concentration). A total of 25 μl of superoxide dismutase (600 units/ml in HHBSS, 12 units/well final concentration) was used as a positive control, all other wells obtained 25 μl of HHBSS. Superoxide anion radical formation was started by addition of 25 μl of TPA (0.55 mg/ml in HHBSS, 55 ng/ml final concentration). After an incubation period of 30 min at 37°C, the reaction was stopped by chilling the plates on ice for 15 min. The plates were centrifuged, and cytochrome *c* reduction was determined in the supernatant 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 (excitation 485/20 nm, emission 620/40 nm; PE Applied Biosystems). Using this method, we could avoid unspecific effects of reducing test compounds that falsify commonly used viability assays based on 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide or 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt bioreduction. The reaction was linear for at least 30 min. $IC_{50}$s were generated from the results of seven to eight serial dilutions of inhibitors tested in duplicate.

Inhibition of LPS-mediated NO production by murine Raw 264.7 macrophages was determined via nitrite levels in culture supernatants by the Griess reaction as described previously (21, 30). Cell numbers were estimated by sulforhodamin B staining (31). Generally, compounds were tested at nontoxic concentrations (cell staining > 50% of LPS-treated control cells). The $IC_{50}$ of curcumin used as a positive control was 7.6 ± 2.3 μM ($n = 3$).

**Antitumor Promoting Effects.** Inhibition of Cox-1 activity was measured by monitoring oxygen consumption during the conversion of arachidonic acid to PGs using a Clark-type $O_2$-electrode (Hansatech Ltd., Kings Lynn, Great Britain; Ref. 21, 32). The reaction mixture contained ~0.2 units Cox-1 in 100 μl of microsome fraction derived from ram seminal vesicles as a crude source of Cox-1 (specific activity 0.2–1 units/mg protein) or 0.23 units of recombinant human Cox-2 (specific activity 43 units/mg protein). For calculation, the rate of $O_2$ consumption was compared with a DMSO control (100% activity). Piroxicam, a nonsteroidal anti-inflammatory drug, was used as positive inhibitory substance for Cox-1 activity with an $IC_{50}$ of 0.35 ± 0.05 μM ($n = 2$). Alternatively, nimesulide, a Cox-2 specific inhibitor, inhibited Cox-2 activity by 52 ± 5.7% ($n = 2$) at a concentration of 50 μM.

Estrogenic and antiestrogenic activity was analyzed in cultured Ishikawa human endometrial adenocarcinoma cells via the E2-dependent induction of ALP activity. Cell culture con-

ditions were according to Ref. 33, 34. Ishikawa cells were trypsinized with 0.05% phenol-red free trypsin/EDTA and plated in 96-well microplates at a density of $2 \times 10^4$ cells/well in 200 $\mu$l of estrogen-free mix (phenol red-free DMEM/F-12 mix (1:1) containing L-glutamate and pyridoxine HCl, supplemented with 5% charcoal-stripped FBS). After 24 h, the medium was replaced by 170 $\mu$l of fresh estrogen-free mix. Test compounds (10 $\mu$l in 10% DMSO, tested in duplicate) or 10 $\mu$l of 10% DMSO (as a negative control, final concentration 0.5%), respectively, and either 20 $\mu$l of estrogen-free mix (for estrogenic activity) or 20 $\mu$l of 50 nM E2 in estrogen-free mix (for antiestrogenic activity) were added to a final volume of 200 $\mu$l, and the plates were incubated for 72 h. Cell viability was measured fluorimetrically by calcein AM hydrolysis as described for the cellular superoxide anion radical assay. The calcein solution was removed immediately, 50 $\mu$l/well 0.5% Triton X in PBS were added, and plates were kept at $-80$°C overnight. After two repetitive freeze-thaw cycles, 100 $\mu$l/well of the ALP substrate 4-MUP (15 $\mu$M 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 4-MUP to fluorescent 4-MU was monitored for 45 min at 37°C (excitation 360/40 nm, emission 460/40 nm). ALP activity and cell viability were determined from the rates of product formation. Relative enhancement of ALP activity indicative of estrogenic activity was computed by comparison with a DMSO solvent control. For calculation of antiestrogenic effects, results were expressed as a percentage in comparison with a control sample treated with DMSO and 5 nM E2. Tamoxifen, a well-known breast cancer chemotherapeutic agent approved for chemoprevention, was used as a positive control substance with an $IC_{50}$ of 0.72 $\pm$ 0.18 $\mu$M ($n = 4$).

**Antiproliferative Mechanisms.** To measure inhibition of DNA synthesis, an *in vitro* bioassay system using recombinant human DNA polymerase $\alpha$-primase complex was established based on Ref. 35, measuring the incorporation of radiolabeled substrate [methyl-$^3$H]dTTP into newly synthesized DNA in a microplate format.[4] The reaction mixture (80 $\mu$l) contained 50 mM Tris-HCl buffer (pH 7.5), 10 mM $MgCl_2$, 2 mM DTT, 10 $\mu$g of activated calf thymus DNA, 10 $\mu$M each of dATP, dCTP, dGTP, and dTTP, and 0.5 $\mu$Ci of [methyl-$^3$H]dTTP. Inhibitors (10 $\mu$l in DMSO) were added (4 serial 4-fold dilutions in a final concentration range of 1.56–100 $\mu$M tested in duplicate), and the reaction was started by the addition of 0.5 units of human recombinant DNA polymerase $\alpha$-primase complex (in 10 $\mu$l buffer). After an incubation period of 30 min, the reaction was stopped by addition of 30 $\mu$l of 0.1 M EGTA solution followed by chilling on ice for 15 min. Aliquots of each reaction mixture were spotted uniformly on DEAE filter mats, kept at an ambient temperature for 15 min, washed four times with 5% aqueous $Na_2HPO_4$ solution, and washed twice with distilled water. Finally, the filtermats were thoroughly dried and subjected to scintillation counting in a Betaplate counter (Wallac). Aphidicolin, a selective inhibitor of DNA polymerase $\alpha$, was used as a reference compound and inhibited the enzyme activity with an $IC_{50}$ of 16.9 $\pm$ 1.7 $\mu$M ($n = 3$). DNA synthesis *in vivo* was determined in MDA-MB-435 human mammary adenocarcinoma cells cultured in RPMI 1640 containing 100 units/ml penicillin G sodium and 100 units/ml streptomycin sulfate supplemented with 10% FBS at 37°C in a 5% $CO_2$ atmosphere. Radiolabeled thymidine incorporation into newly synthesized DNA was measured as described previously (17).

For flow cytometric analyses of cell cycle distribution, MDA-MB-435 cells were plated in 60-mm tissue culture dishes ($5 \times 10^5$ cells in 5-ml medium). After a preincubation period of 24 h, cells were treated with 5, 10, and 20 $\mu$M XN for 24 h. Cells were harvested by trypsinization, washed three times with PBS, and passed through a 14 gauge injection needle into ice-cold ethanol. Cell cycle distribution was determined by fluorescence-activated cell sorting analyses after propidium iodine staining by standard techniques (36, 37). The degree of cells undergoing apoptosis after a 48-h treatment with 25 $\mu$M XN was estimated from the percentage of cells with a DNA content $<1$ (sub-$G_1$ peak) indicative of fragmented DNA. Induction of terminal cell differentiation was measured in HL-60 promyelocytic leukemia cell culture. HL-60 cells were maintained in RPMI 1640 supplemented with 10% FBS at 37°C in a 5% $CO_2$ atmosphere. Log-phase cells with a population doubling time of 14–16 h were used for experiments. Serial 2-fold dilutions of compounds (dissolved in DMSO, final concentration 0.1%) in a final concentration range of 0.2–12.5 $\mu$M were prepared in 24-well plates using 1 ml of RPMI/well. Control wells obtained the same amount of solvent. Subsequently, 1 ml of the cell suspension was added to the wells ($2 \times 10^4$ cells/ml; final cell concentration $1 \times 10^4$ cells/ml). After 96 h, the experiment was evaluated. Cell numbers were counted using a Casy 1 TTC flow-cytometer (Schärfe System). The proliferation of treated cells was expressed as a percentage in comparison with the solvent control. Induction of differentiation to morphological and functional mature granulocytes and monocytes/macrophages was determined by NBT reduction after TPA challenge and by expression of nonspecific/specific acid esterase essentially as described previously (38). DMSO (1%) as a well-described differentiation inducer in HL-60 cell culture was included as a positive control.

**MMOC.** The identification of potential inhibitors of DMBA-induced preneoplastic lesion formation in mammary gland organ culture was performed as described earlier (39). The incidence of forming lesions (the percentage of glands with mammary lesions of the total number of glands per group) in the compound-treated groups was compared with that of the DMBA-control group, and the percentage of inhibition was calculated. To evaluate antiproliferative effects, the organ culture (without addition of DMBA) was terminated after the initial growth period of 10 days, and compound-treated and control glands were fixed and stained as described. Pictures were acquired using an Axiocam digital camera (Zeiss Vision) and a stereomicroscope (Olympus). Animal care was in accordance with institutional guidelines.

[4] M. Spaeth, N. Frank, H. Schiffter, I. Neumann, and C. Gerhauser. Inhibition of DNA polymerase $\alpha$ by potential cancer chemopreventive agents, manuscript in preparation.

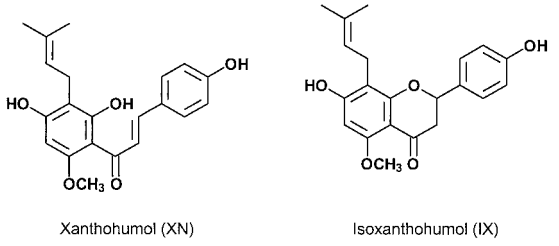


*Fig. 1*. Chemical structures. XN (2′,4,4′-trihydroxy-3′-prenyl-6′-methoxychalcone) and IX (5-*O*-methyl-8-prenylnaringenin).

**Statistical Analysis.** For statistical analysis, means were compared using a two-sided Student's *t* test.

## Results

**Isolation of XN and IX from Hop.** A commercially available hop extract was subjected to bioassay-guided fractionation. A series of prenylated chalcones and flavonoids, including XN and IX (Fig. 1), was isolated by size exclusion and vacuum liquid chromatography, structurally characterized and tested to define chemopreventive mechanisms. Of all hop constituents tested, XN was identified as the most promising agent with multiple hitherto unknown activities indicative of cancer preventive potential at various stages of tumor development. The structural characterization of XN and IX, first isolated from hop in 1913 by Power *et al.* (summarized in Ref. 9), was in agreement with data in the literature (19, 20).

**Anti-initiating Mechanisms: Modulation of Carcinogen Metabolism.** Modulation of enzymes involved in metabolic activation (Phase 1), conjugation (Phase 2), and excretion of carcinogens is one of the best investigated mechanisms of chemopreventive agents and is of importance in the prevention of tumor initiation (40). We selected Cyp1A, which is involved in the activation of procarcinogens to ultimate carcinogens, to analyze potential to inhibit Phase 1 enzymes. Both XN and IX were identified as potent inhibitors of Cyp1A activity *in vitro*, with $IC_{50}$s of 0.022 ± 0.002 μM for XN and 0.30 ± 0.13 μM for IX ($n = 2$; Fig. 2*A*). XN demonstrated competitive inhibition with respect to the substrate CEC, determined by Lineweaver-Burk (data not shown), Dixon, and Cornish-Bowden plots (Fig. 2*B*) of the results of kinetic experiments, and the inhibitory constant ($K_i$) was determined as 0.041 μM. In agreement with Ref. 25, competitive inhibition was also reflected by a decrease in $IC_{50}$s obtained with XN with decreasing substrate concentrations (data not shown).

Phase 2 enzymes generally conjugate activated xenobiotics to endogenous ligands. QR activity, which is induced coordinately with other Phase 2 enzymes like glutathione *S*-transferases and contributes to the detoxification of quinones, was investigated in murine hepatoma cell culture as a representative for Phase 2 enzymes. After a 48-h treatment period, XN was found to induce the specific activity of QR in a dose-dependent manner with a CD value of 1.67 ± 0.23 μM ($n = 2$; Fig. 2*C*). As indicated by a reduction in QR activity, XN was cytotoxic at concentrations above 6.25 μM. The $IC_{50}$ of cell viability was determined as 7.4 ± 1.4 μM ($n = 2$). Loss of

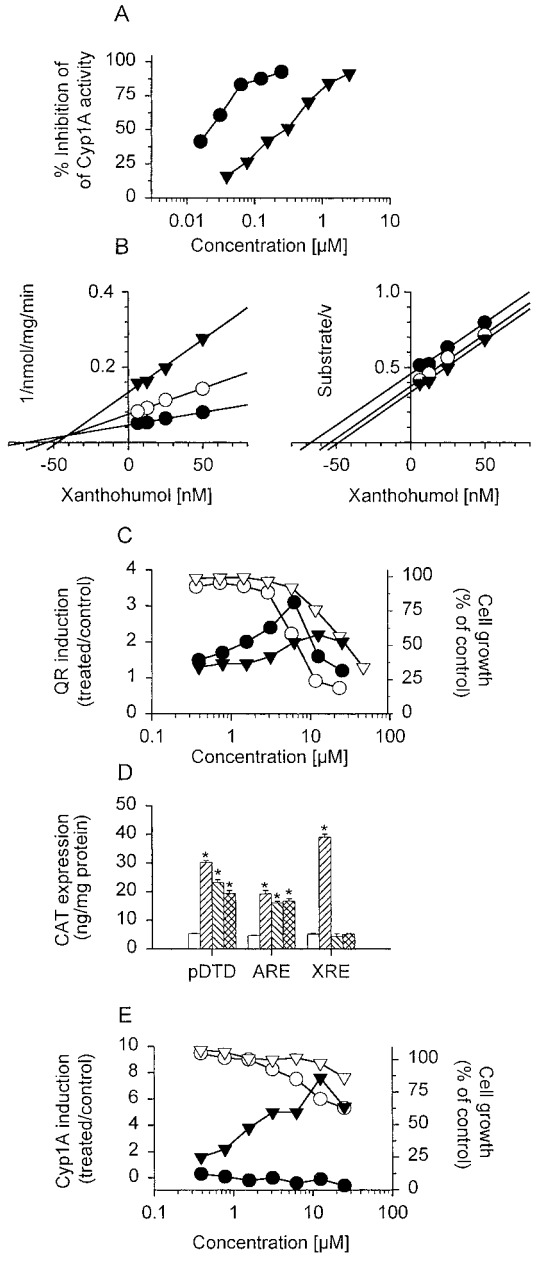


*Fig. 2*. Anti-initiating mechanisms: modulation of carcinogen metabolism. *A*, dose-dependent inhibition of Cyp1A enzymatic activity by XN (●) and IX (▼), respectively, measured in β-NF-induced H4IIE rat hepatoma cell homogenates by dealkylation of CEC to fluorescent CHC. Activity of β-NF-induced controls: 23 ± 2 nmol/min/mg of protein ($n = 4$). *B*, competitive inhibition of Cyp1A activity by XN. Dixon (*left*) and Cornish Bowden plots (*right*) of the results of kinetic experiments using 2.5 (▼), 5 (○), or 10 μM (●) CEC, respectively, as a substrate. *C*, induction of QR activity in Hepa 1c1c7 cell culture by XN (●) or IX (▼). Effects of XN (○) or IX (▽) on cellular growth were estimated from crystal violet staining. Specific activities of untreated controls were as follows: XN: 87 ± 6 nmol/min/mg protein; IX: 63 ± 3 nmol/min/mg protein. *D*, quantification of CAT expression in Hepa 1c1c7 cells transiently transfected with QR-CAT constructs pDTD-1097 CAT (*pDTD*), QR XRE-CAT (*XRE*), or QR ARE-CAT (*ARE*) and treated with β-NF (2 μM, ▨), sulforaphane (5 μM, ▧), XN (5 μM, ▩), or 0.1% DMSO as a solvent control (□) for 24 h. ∗, mean significantly different from control ($P < 0.0001$) using the Student's *t* test with $n = 3$. *E*, induction of Cyp1A activity in Hepa 1c1c7 cell culture by XN (●) or IX (▼). Effects of XN (○) or IX (▽) on cellular growth were estimated from crystal violet staining. Specific activities of untreated controls: 0.22 ± 0.08 nmol/min/mg protein ($n = 4$).

the $\alpha,\beta$-unsaturated keto-group by cyclization to the flavanone IX reduced both the inducing potential as well as toxic effects. For IX, we determined a CD value of 6.5 $\pm$ 1.5 $\mu$M with a concomitant $IC_{50}$ of 29.9 $\pm$ 1.9 $\mu$M ($n = 2$).

To additionally investigate the mode of enzyme induction and to distinguish monofunctional and bifunctional inducer mechanisms (*i.e.,* selective induction of Phase 2 enzymes *versus* simultaneous induction of Phase 1 and 2 enzymes), Hepa 1c1c7 cells were transiently transfected with plasmid constructs containing various portions of the 5′ regulatory region of the rat *QR* gene linked to the *CAT* structural gene. Sulforaphane and $\beta$-NF, tested as monofunctional and bifunctional reference compounds, as well as XN significantly ($P < 0.0001$) induced CAT levels after transfection with the pDTD-1097CAT construct (containing all of the known regulatory elements of the rat *QR* gene) or the ARE-CAT construct. However, only $\beta$-NF induced CAT expression when the XRE-CAT construct, as a target for an aryl hydrocarbon receptor-mediated bifunctional mechanism of induction, was used for transient transfection (Fig. 2*D*). These findings were additionally confirmed by directly measuring Cyp1A induction in intact Hepa 1c1c7 cells. Treatment with IX for 24 h in a concentration range of 0.4–25 $\mu$M led to a maximal 8-fold induction of Cyp1A activity, whereas XN was inactive with this respect, indicative of a preferential monofunctional mechanism of induction (Fig. 2*E*). Because of a shorter incubation period and a higher initial cell number, both compounds were less toxic under these experimental conditions than in the QR induction assay (Fig. 2*C*).

**Antioxidant Capacity.** Overproduction of ROS during conditions of oxidative stress results in DNA damage and contributes to tumor initiation. Additionally, elevated levels of ROS are associated with tumor promotion and might ultimately lead to carcinogenesis (41). Also, excessive production of NO, a reactive nitrogen oxide species, during infection and chronic inflammation is thought to initiate cellular injury and carcinogenesis, *e.g.,* via nitrosative deamination of DNA bases and induction of lipid peroxidation (42). Therefore, in addition to anti-initiating mechanisms by modulation of carcinogen metabolism, XN and IX were tested for anticarcinogenic potential by scavenging of physiologically relevant ROS, including hydroxyl-, peroxyl-, and superoxide anion radicals. In the ORAC assay, XN was 8.9-fold and 2.9-fold more potent than the reference compound Trolox in scavenging hydroxyl- and peroxyl radicals, respectively, at a concentration of 1 $\mu$M. IX was equally effective as Trolox in inactivating hydroxyl radicals and even more potent than XN in peroxyl radical scavenging, especially at a concentration of 5 $\mu$M (Fig. 3*A*). In the xanthine oxidase system used to generate superoxide anion radicals, XN displayed a $SC_{50}$ of 27.7 $\pm$ 4.9 $\mu$M ($n = 2$; Fig. 3*B*). Concomitant detection of uric acid proved that the result was not falsified by direct inhibition of xanthine oxidase enzymatic activity (data not shown). Furthermore, XN inhibited superoxide anion radical production by TPA stimulation of differentiated HL-60 cells with an $IC_{50}$ of 2.6 $\pm$ 0.4 $\mu$M ($n = 2$). This indicated that in addition to direct superoxide anion radical scavenging potential, XN might inhibit the signal transduction cascade induced by TPA. IX was inactive in both systems at concentrations

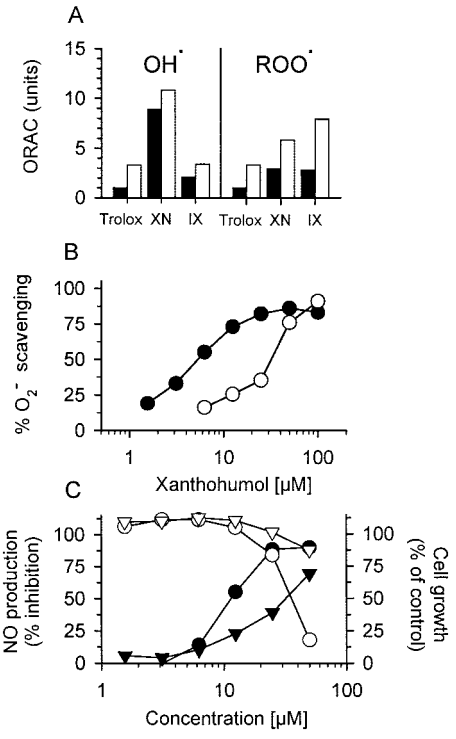


*Fig. 3.* Anti-initiating mechanisms: antioxidant activities. *A*, hydroxyl- and peroxyl radical scavenging capacity determined in a microplate ORAC assay. Protection of $\beta$-PE fluorescence by Trolox (as a reference substance), XN, and IX, respectively, was tested at 1 $\mu$M (■) and 5 $\mu$M (□). One ORAC unit equals the net protection provided by 1 $\mu$M Trolox. *B*, scavenging of superoxide anion radicals *in vitro* by XN (○) in the xanthine oxidase system (NBT reduction of control: −superoxide dismutase: 335 $\pm$ 10 nmol/min/mg protein; +superoxide dismutase: 57 $\pm$ 10 nmol/min/mg protein; $n = 4$). Determination of superoxide anion radical scavenging activity in TPA-stimulated differentiated HL-60 cells treated with various concentrations of XN (●). Control levels were 11.5 $\pm$ 1.4 nmol reduced cytochrome *c*/30 min/2 $\times$ $10^5$ cells ($n = 4$). *C*, inhibition of LPS-mediated NO production in Raw 264.7 murine macrophages treated with XN (●) or IX (▼). Nitrite levels in unstimulated controls: 8.4 $\pm$ 0.9 nmol nitrite/mg protein; after LPS stimulation: 77.7 $\pm$ 7.7 nmol nitrite/mg ($n = 4$). Effects on cell growth by XN (○) or IX (▽) were measured by sulforhodamin B staining.

<100 $\mu$M. When Raw 264.7 murine macrophages were stimulated with bacterial LPS to enhance inducible nitric oxide synthase expression and NO production, nitrite levels (as a measure of NO) in cell culture supernatants were dose dependently inhibited by XN with an $IC_{50}$ of 12.9 $\pm$ 1.2 $\mu$M ($n = 3$). Concomitantly, cell growth was reduced with an $IC_{50}$ of 43.5 $\pm$ 5.7 $\mu$M. IX was also active, with an $IC_{50}$ of 21.9 $\pm$ 2.6 $\mu$M ($n = 2$) and only marginally affecting cell growth (Fig. 3*C*).

**Antitumor-promoting Effects.** Excessive production of PGs, *i.e.,* hormone-like endogenous mediators of inflammatory processes, has been associated with carcinogenesis. Elevated levels of PGs are often found in tumor tissue, stimulate cell proliferation, and initiate formation of new blood vessels (angiogenesis) essential for tumor growth (43). For the first time, XN was characterized as an effective anti-inflammatory agent. It dose dependently inhibited the activity of the constitutive form of cyclooxygenase Cox-1 with an $IC_{50}$ of 16.6 $\pm$ 1.8 $\mu$M ($n = 2$). XN also inhibited the activity of the inducible Cox-2, which is linked to carcinogenesis, with

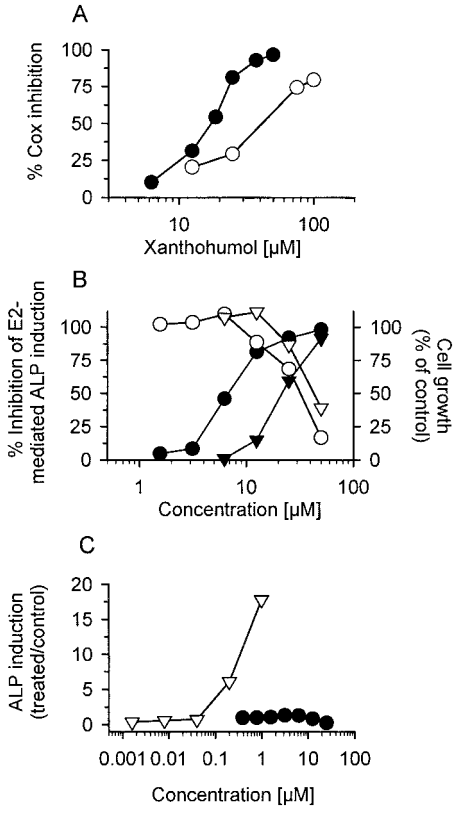


*Fig. 4.* Antitumor promoting mechanisms. *A*, XN-mediated inhibition of oxygen consumption during *in vitro* PG formation by Cox-1 (●) or Cox-2 (○). *B*, antiestrogenic potential of XN (●) and IX (▼) in Ishikawa cell culture. Effects on cell growth by XN (○) or IX (▽) were determined by calcein AM hydrolysis. Control levels of ALP activity in untreated Ishikawa cells: 0.61 ± 0.07 pmol 4-MU/min/mg protein ($n = 4$); after stimulation with 5 nM E2: 6.0 ± 0.68 pmol 4-MU/min/mg protein ($n = 4$). *C*, induction of ALP activity as a measure of estrogenic properties of XN (●) and IX (▽). Growth inhibitory effects were comparable with the results in *B*. Control levels of unstimulated cells: 0.13 fmol 4-MU/min, normalized for the conversion rate of calcein AM in arbitrary fluorescence units/min.

an $IC_{50}$ of 41.5 μM. Although it can be assumed that inhibition of enzymatic activities will result in reduced PG levels, the effect of XN on intracellular PG production has not been investigated thus far. IX was inactive with respect to both enzymes ($IC_{50} > 100$ μM; Fig. 4*A*). Similar to PGs, hormones like E2 are regarded as endogenous tumor promoters, stimulate cell growth via interaction with estrogen receptors, and increase the risk for breast and uterine cancer (44). Therefore, we analyzed antiestrogenic potential in the Ishikawa human endometrial adenocarcinoma cell line. Enhancement of ALP activity allowed the assessment of intrinsic estrogenic activity of test compounds. Antiestrogenic effects were determined by cotreatment with E2 and inhibitors. Both XN and IX dose dependently inhibited E2-mediated induction of ALP activity with $IC_{50}$s of 6.6 ± 0.2 and 20.9 ± 1.2 μM ($n = 2$), respectively (Fig. 4*B*) and weakly inhibited cell growth with $IC_{50}$s of 34.5 ± 0.1 μM (XN) and 45.7 ± 1.2 μM (IX; $n = 2$). Because hop has been reported to possess estrogenic properties, the compounds were also tested without prior E2 stimulation to assess estrogenic potential. XN was identified as a pure antiestrogen, whereas IX induced ALP activity dose dependently with a maximum 17-fold induction at a concentration of 1.0 μM (Fig. 4*C*). Importantly, 8-PN, the 5-demethylated analogue of IX, was found to induce ALP activity in Ishikawa cell culture almost as effectively as E2. At a concentration of 0.004 μM, 8-PN and E2 enhanced ALP activity 19- and 30-fold, respectively, in comparison with the untreated control (data not shown). Therefore, we currently cannot exclude that a small fraction of IX is demethylated at the 5-position to provide 8-PN, which would then mimic the observed estrogenic response by IX. A free 5-hydroxyl group has been proposed to be essential for estrogen receptor binding of 8-PN (45).

**Antiproliferative Mechanisms to Inhibit Tumor Progression.** Characteristic of tumor cells in the progression phase is uncontrolled cell proliferation independent of hormone- or growth factor stimulation and impairment of mechanisms like apoptosis (programmed cell death) and terminal cell differentiation, which are important in regulating tissue homeostasis (46, 47). To assess antiproliferative potential of XN and IX, we first investigated their influence on DNA synthesis. In an *in vitro* test system, XN inhibited the activity of human DNA polymerase α, the only eukaryotic polymerase that can initiate DNA synthesis *de novo* with an $IC_{50}$ of 23.0 ± 3.5 μM ($n = 3$). IX was less active and demonstrated 43.8% inhibition at a concentration of 100 μM (Fig. 5*A*). A similar effect was observed in cell culture. After treatment of MDA-MB-435 mammary adenocarcinoma cells with XN for 48 h, thymidine incorporation into newly synthesized DNA was 59.6% inhibited by 25.0 μM XN (Fig. 5*B*). Consistently, flow cytometric analyses revealed a significant dose-dependent accumulation of MDA-MB-435 cells in S phase of the cell cycle ($P \leq 0.01$) when treated with XN for 24 h at concentrations of 5, 10, and 20 μM. The percentage of cells in the $G_0$-$G_1$ phase decreased from 48.7 ± 2.1% in the control group to 36.6 ± 4.1% and 30.0 ± 5.9% after treatment with 10 and 20 μM XN, respectively, whereas the percentage of cells in the S phase increased from 45.0 ± 3.4% in the control group to 58.9 ± 4.3% and 65.8 ± 5.3% after XN treatment (Fig. 5*C*). In addition, after a 48-h treatment period with 25 μM XN, 14.9% of the attached cell population underwent apoptosis, detected by a sub-$G_1$ peak in histograms of flow cytometric experiments (data not shown). As an additional mechanism to control cell proliferation of (pre-)neoplastic cells, induction of terminal cell differentiation was measured in HL-60 human promyelocytic leukemia cell culture. HL-60 cells undergo growth arrest while they terminally differentiate. Consequently, cell growth determined by cell counting was reduced by XN with an $IC_{50}$ of 3.7 μM. This growth inhibitory effect was accompanied by an induction of differentiation markers, *i.e.,* treatment with 3.1 and 6.25 μM XN resulted in expression of nonspecific acid esterase as an indicator for differentiation along the monocytic-macrophagic lineage in 42.4 and 44.8%, respectively, of viable cells (Fig. 5*D*). Because of problems in solubility, IX was tested only up to a concentration of 12.5 μM, and the observed effects were marginal.

**Inhibition of Preneoplastic Lesions in MMOC.** To avoid identification as a false positive lead, *i.e.,* a compound that

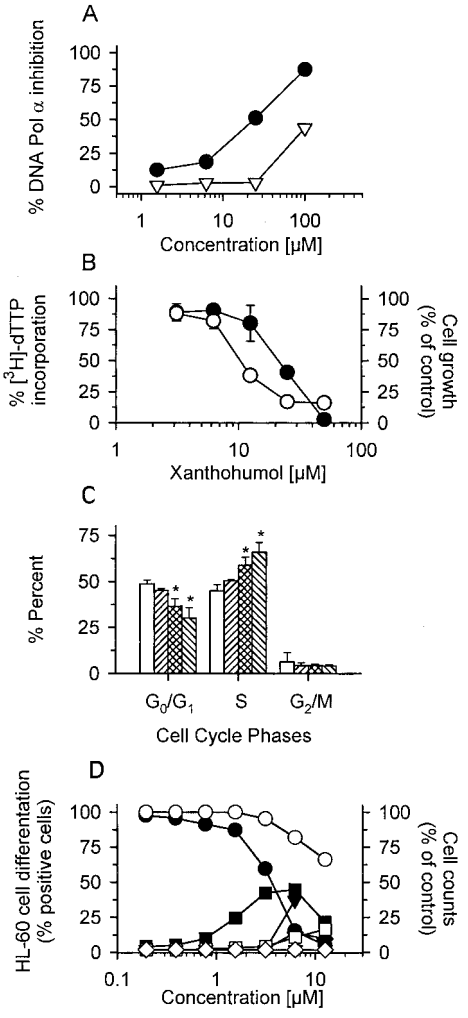


*Fig. 5*. Anticarcinogenic mechanisms in the progression phase. *A*, inhibition of human recombinant DNA polymerase α activity *in vitro* by XN (●) and IX (▽). *B*, inhibition of [ $^3$H]dTTP incorporation into newly synthesized DNA in cultured MDA-MB-435 breast cancer cells treated with various concentrations of XN (●). Levels of untreated controls: 171.2 ± 11.9 cpm/μg protein ($n$ = 4). Total protein was measured to determine an influence on cell viability (○). *C*, cell cycle distribution of MDA-MB-435 breast cancer cells determined by fluorescence-activated cell sorting analyses. Cells were treated for 24 h with 0.1% DMSO (solvent control, □), 5 μM XN (▨), 10 μM XN (▩) or 20 μM XN (▧), respectively. ∗, mean significantly different from control ($P \leq 0.01$) using the Student's $t$ test with $n$ = 3. *D*, induction of cell differentiation in HL-60 human promyelocytic leukemia cells. Effects on cell numbers (●, XN; ○, IX), NBT reduction as a measure of differentiation toward the granulocyte lineage (▼, XN; ▽, IX), and expression of nonspecific (■, XN; □, IX) and specific acid esterase (✦, XN; ✧, IX) as an indication of monocyte/macrophage cell differentiation after treatment with test compounds for 72 h.

shows activity *in vitro* but fails to inhibit carcinogenesis *in vivo*, XN was tested in MMOC (39) as a link between short-term *in vitro* and long-term *in vivo* carcinogenesis models. Explanted mammary glands were maintained in culture for a total of 24 days. An initial proliferation phase (10 days) stimulated by mammotrophic hormones was followed by a regression phase (14 days) after hormone withdrawal. Preneoplastic lesions were induced by DMBA treatment on day 3. Importantly, XN at nanomolar concentrations inhibited lesion formation with an $IC_{50}$ of 0.02 μM (Table 1). Resveratrol, a tri-hydroxy stilbene found in grapes and red wine (32), was tested as a reference compound with an $IC_{50}$ of 4.2 μM (Table 1). When the organ culture experiment was terminated after the initial growth phase (no DMBA treatment on day 3), lobulo-alveolar structures observed in control glands were mainly absent in XN-treated glands (Fig. 6). Because results in the MMOC model have been shown previously to demonstrate good correlation with the outcome of long-term carcinogenesis models (48), we have initiated an *in vivo* study to investigate the efficacy of XN in a rat mammary tumor model.

*Table 1* Effect of XN on the incidence of DMBA-induced lesions in Balb/c MMOC

| Treatment group | No. of glands with lesions/ total no. of glands | % incidence[a] | % inhibition[b] |
|---|---|---|---|
| None (control) | 28/35[c] | 80 | n.d.[d] |
| XN (μM) | | | |
| 0.001 | 12/18 | 66.7 | 16.3 |
| 0.01 | 8/19 | 42.1 | 47.4 |
| 0.1 | 6/20 | 30 | 62.5 |
| 1.0 | 2/20 | 10 | 87.5 |
| None (control) | 18/20 | 90 | n.d. |
| Resveratrol (μM) | | | |
| 0.1 | 18/20 | 90 | 0 |
| 1.0 | 13/17 | 76 | 15.5 |
| 10.0 | 7/21 | 33 | 63.3 |
| 100.0 | 6/23[e] | 26 | 71.1 |

[a] (No. of glands with lesions/no. of glands treated) × 100.
[b] 1 − (% incidence in the treated group/% incidence in the control group) × 100.
[c] Results of two identical experiments were pooled.
[d] n.d., not determined.
[e] Some signs of toxicity were visible.

## Discussion

Activity-guided fractionation of a hop extract led to the isolation of XN and of its cyclization product, the flavanone IX, as potential cancer chemopreventive agents. Although hop constituents have been previously reported to modulate carcinogen metabolism and to act by cytotoxic/cytostatic mechanisms, a demonstration of chemopreventive activity in animal models and of potential value for chemoprevention in humans is missing.

*In vivo* efficacy of selected chalcones as chemopreventive agents against pulmonary and oral carcinogenesis has been demonstrated in a limited number of studies (49, 50). Interestingly, the chalcone structure of XN is modified by substitution with a prenyl side chain. Prenylation is an important posttranslational modification of proteins, *e.g.,* the Ras oncoprotein, and results in higher lipophilicity and targeting of the modified protein to the cell membrane (51). It is tempting to speculate that likewise, prenylation might also influence solubility, cellular uptake, and subcellular localization of XN and thus affect its biological activities.

With respect to inhibition of tumor initiation, XN was identified as a potent inhibitor of Cyp1A activity and as an inducer of QR activity in mouse hepatoma cell culture. Transient

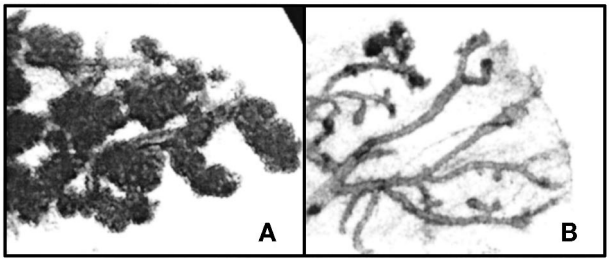


*Fig. 6*. Inhibition of lobulo-alveolar growth in mouse mammary glands. Mammary glands were incubated with insulin, prolactin, aldosterone and hydrocortisone for 10 days (no treatment with DMBA), fixed with formalin, and stained with alum carmine. Normal lobulo-alveolar structures are shown in *A* after treatment of mammary glands with 0.1% DMSO as solvent control. *B* represents a partial view of a mammary gland incubated with 1.0 $\mu$M XN. Gland morphology was monitored using computer-based digital image analysis.

transfection experiments indicated that QR induction was mediated by a selective activation of transcription factors interacting with the ARE in the promoter region of the *QR* gene in an aryl hydrocarbon receptor-independent mechanism. The observed profile of modulatory effects on carcinogen-metabolism represents a favorable combination of activities for cancer chemoprevention as *e.g.,* Cyp1A might contribute to the activation of heterocyclic and polycyclic aromatic hydrocarbons, including DMBA, to ultimate carcinogens. Accordingly, Miranda *et al.* (52) described that prenylflavonoids from hop inhibited the mutagenic activation of the food-derived heterocyclic amine 2-amino-3-methylimidazo[4,5-f]quinoline by Cyp1A2.

As a preventive approach during tumor initiation and tumor promotion, we have identified XN as an effective antioxidant with capacity to scavenge ROS, including hydroxyl-, peroxyl-, and superoxide anion radicals. Earlier reports have indicated that prenylated flavonoids from hop and beer were more efficient in preventing liver microsomal lipid peroxidation than nonprenylated flavonoids, including quercetin (53), providing additional information that prenylation represents a crucial structural modification of XN that will influence chemopreventive potential.

Elevated production of NO as a result of, *e.g.,* chronic inflammatory conditions or bacterial infections, also can be regarded as a causative factor for both tumor initiation and tumor promotion. Using the Raw 264.7 macrophage model, we demonstrated that XN prevented NO release after LPS stimulation of cultured cells. In this system, LPS treatment leads to a transcription factor nuclear factor $\kappa$B-dependent elevated expression of proinflammatory proteins, including inducible nitric oxide synthase, Cox-2, and tumor necrosis factor $\alpha$. In view of the fact that activation and nuclear translocation of nuclear factor $\kappa$B is dependent on the intracellular redox status (54), we assume that the antioxidant activity of XN might contribute to the inhibition of LPS-induced production of NO, similar to the activity of Broussochalcone A, a prenylated antioxidative chalcone isolated from *Broussonetia papyrifera* Vent (55). Furthermore, because of the coordinate pattern of induction, Cox-2 and tumor necrosis factor $\alpha$ induction involved in tumor promotion might also be susceptible to XN inhibition as we have demonstrated with sulforaphane, an isothiocyanate from broccoli (30). In a recent report, the transcription of Cox-2 was suggested to be responsive to hormonal regulation (reviewed in Ref. 56) and E2, as a steroidal hormone, was shown to stimulate the secretion of PGs $E_2$ and $F_2$ in human mammary tissue (57). Because we have identified XN as a potent antiestrogen, this might add indirectly to anti-inflammatory and antitumor promoting effects apart from the direct inhibition of Cox-1 and Cox-2 enzymatic activity described in this report.

As a first direct proof of its chemopreventive potential, XN at nanomolar concentrations prevented the development of DMBA-induced nodule-like alveolar lesions in MMOC. In this model, prepubertal mice are primed with steroidal hormones to induce mammary gland proliferation *in vivo*. After explantation, the glands are initiated with DMBA, and lobulo-alveolar growth is promoted with aldosterone and hydrocortisone. Keshava *et al.* (58) have demonstrated that early estrogen exposure of mammary epithelial cells resulted in preneoplastic changes and in increased susceptibility to environmental carcinogens. Thus, exposure of the glands to antiestrogenic XN before DMBA treatment might lower sensitivity toward the carcinogen as an anti-initiating mechanism in addition to XN-mediated prevention of DMBA metabolism by Cyp1A inhibition and enhanced detoxification by Phase 2 induction. However, because preneoplastic lesion formation in the standard MMOC protocol is independent of estrogenic stimulation and, *e.g.,* tamoxifen failed to prevent lesion development, antiestrogenic properties seem to contribute little to the strong inhibitory potential of XN in this model. To further explore the preventive mechanism, we modified the experimental protocol and fixed and stained solvent- or XN-treated glands after the initial growth period of 10 days. We could demonstrate that lobulo-alveolar structures were mainly absent in XN-treated glands, indicative of an antiproliferative mode of action independent of carcinogen-induced growth promoting effects. Consequently, we found that XN inhibited DNA polymerase $\alpha$ activity *in vitro*, as well as thymidine incorporation into newly synthesized DNA *in vivo*, and caused a cell cycle arrest in S phase in the estrogen receptor-negative MDA-MB-435 mammary adenocarcinoma cell line. Thus, XN might prevent transformation of mammary epithelial cells in mammary organ culture by inhibition of carcinogen-induced and mammotrophic hormone-promoted proliferation at the level of DNA synthesis. Finally, XN could induce terminal cell differentiation in transformed cells as a mechanism of prevention effective in late stages of carcinogenesis. A combination of all mechanisms (*n.b*. the concept of combination cancer chemoprevention; Ref. 59) could lead to additive or synergistic amplification of chemopreventive potential as we have observed in the MMOC model ($IC_{50}$ in the nM range) in comparison with the results from short-term bioassays ($IC_{50}$s mostly in the $\mu$M range). Therefore, XN could offer a distinct advantage over compounds acting on specific targets like, *e.g.,* rotenoids, which are known inhibitors of mitochondrial respiration and inhibit the induction of ornithine decarboxylase during tumor promotion (60).

In addition to confirming cancer preventive potential in animal models, determination of bioavailability will be of im-

portance for additional development of XN as a chemopreventive agent (4). Recent *in vitro* metabolism studies using rat and human liver microsomes have yielded four metabolites of XN, including XN with an additional hydroxyl group and dehydro-cycloXN, generated by a proposed mixed enzymatic and chemical biotransformation pathway (61). Furthermore, *in vitro* glucuronidation has provided two monoglucuronides of XN (62). XN as a prenylated chalcone is part of the hop resin and contributes to the bitter principle of hop in beer. Although XN represents one of the major secondary metabolites in hop, the amount of XN in beer is constantly reduced during the beer-brewing process because of cyclization to IX (63). We have determined the concentration of XN in a series of German beers as 0.08 ± 0.03 mg/l ($n = 34$) by solid phase extraction and subsequent high pressure liquid chromatography. On a molar basis, these levels are equal to 0.23 ± 0.08 μM. Although low in hop, the content of IX in beer is about 10- to 20-fold higher than that of XN, depending on the type of beer. In Pilsener type beers ($n = 17$), we detected IX-levels up to 1.6 mg/liter in normal brews (mean concentration, 0.74 ± 0.5 mg/liter) and 40% reduced levels in dealcoholized or light beers (0.44 ± 0.35 mg/liter; $n = 6$). Wheat beers also contained lower concentrations in the range of 0.4 ± 0.24 mg/liter ($n = 7$).[5] Overall, it can be concluded that the amounts of prenylated flavonoids present in a maximally recommended daily amount of up to 0.5 liter of beer (consistent with moderate alcohol uptake) might not be sufficient to achieve the activities described in this report. Because extent and rate of uptake of XN in and elimination from the body are thus far unknown, we are currently investigating *in vivo* biotransformation and oral bioavailability of XN in rats. Beside analyses on kinetics and whole body distribution, isolation and identification of metabolites and analyses of their potential biological activities will be of major interest. Although these additional studies and demonstrations of *in vivo* efficacy in a rat mammary tumor model are ongoing, our findings support the use of XN as a novel broad-spectrum chemopreventive agent and provide evidence for promising applications of XN and hop products with respect to cancer prevention; clinical investigations in humans are therefore warranted.

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