

# Xanthohumol, a prenylated chalcone derived from hops, inhibits proliferation, migration and interleukin-8 expression of hepatocellular carcinoma cells

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**Abstract.** Xanthohumol, the major prenylated chalcone found in hops, is well known to exert anti-cancer effects, but information regarding the impact on hepatocellular carcinoma (HCC) cells and potential adverse effects on non-tumorous hepatocytes is limited. Here, we show that xanthohumol at a concentration of 25  $\mu\text{M}$  induced apoptosis in two HCC cell lines (HepG2 and Huh7). Furthermore, xanthohumol repressed proliferation and migration, as well as TNF induced NF- $\kappa\text{B}$  activity and interleukin-8 expression in both cell lines at even lower concentrations. In contrast, xanthohumol concentrations up to 100  $\mu\text{M}$  did not affect viability of primary human hepatocytes *in vitro*. In summary, our data showed that xanthohumol can ameliorate different pro-tumorigenic mechanisms known to promote HCC progression, indicating its potential as promising therapeutic agent that selectively affects cancer cells.

## Introduction

Xanthohumol (XN) is the principal prenylated chalcone of the female inflorescences (hop cones, hops) of the hop plant *Humulus lupulus* L. XN has been shown to have several biological activities, and its anti-tumorigenic effect on different types of cancer cells including colon, ovarian, breast and prostate cancer or fibrosarcoma is most extensively investigated (1-6). These studies revealed that XN inhibits tumor growth and angiogenesis and induces apoptosis of tumor cells. However, so far only limited information is available regarding the effects of XN on hepatocellular carcinoma (HCC) or HCC cells, respectively.

HCC is one of the most frequent malignant tumors worldwide with an incidence still rising (7-9). Morbidity and

mortality correlate directly with surgical resectability of the primary tumor. However, outcome is mostly poor, since most patients are diagnosed at an advanced stage, and only 10-20% of HCCs can be resected completely (10,11). Hence, limited treatment options and the poor prognosis of HCC emphasize the importance of developing novel therapeutics for this highly aggressive tumor.

Hops are used to add bitterness and flavor to beer, and therefore, beer is the major dietary source of XN. Due to the poor solubility in aqueous solutions the average content of XN in beer is most likely not sufficient to produce a protective effect in humans. However, XN is present in hops in concentrations around 1% of the dry weight. In light of the known biological effects of XN on tumors of different entities it appears worthwhile to further study the potential of XN to ameliorate HCC development and progression. In addition to its anti-tumorigenic efficacy every potential therapeutic compound has to be tested regarding potential adverse effects on the liver, as the main metabolizing organ. This is even more important in case of HCC since this tumor develops in the majority of cases in a cirrhotic liver where the metabolic capacity is restricted and hepatocytes are fragile (7-9). Therefore, the aim of the present study was to analyze the effects of XN on HCC cells as well as on primary human hepatocytes.

## Materials and methods

**Cell isolation and culture.** HepG2 (ATCC HB-8065) and Huh7 (JCR B0403) cells were cultured as described (12,13). Isolation and culture of primary human hepatocytes (PHH) were performed as described previously (12-14). Human liver tissue for cell isolation was obtained from the charitable state controlled foundation HPCR, with informed patient consent and approved by the local Ethics Committee.

**Chemicals.** Xanthohumol was obtained from Alexis Biochemicals (Lausen, Switzerland) with a purity  $\geq 98\%$  determined by HPLC. For *in vitro* experiments XN was dissolved in DMSO and added to cell culture at the indicated concentrations. Samples indicated as controls contained vehicle (DMSO) only. TNF was obtained from R&D Systems (Wiesbaden-Nordenstadt, Germany), all other chemicals from Sigma Pharmaceuticals (Hamburg, Germany).

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**Key words:** xanthohumol, chalcone, hepatocellular carcinoma, apoptosis, proliferation, migration

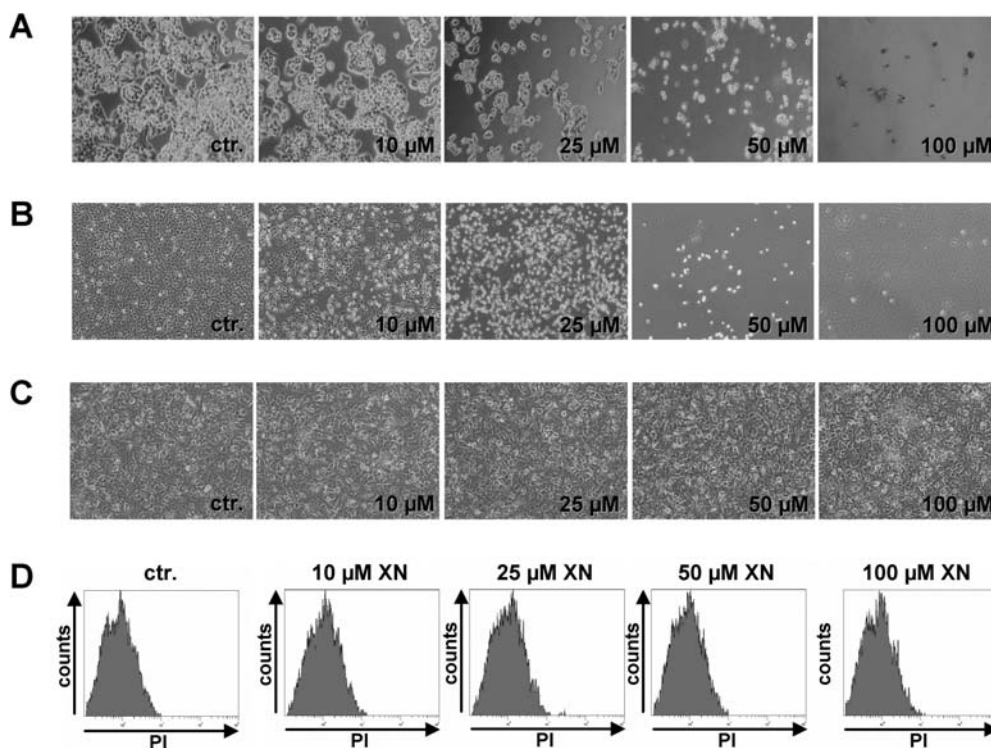


Figure 1. Effect of Xanthohumol on viability of HCC cells and primary hepatocytes. Representative phase-contrast images of (A) HepG2 and (B) Huh7 hepatocellular carcinoma cell line cultures and (C) cultures of primary human hepatocytes (PHH) after 24 h incubation with the indicated concentrations of xanthohumol (XN). (D) Flow cytometry analysis of propidium iodide (PI) stained PHH incubated with different XN concentrations for 24 h. Experiments were performed in triplicate and were repeated at least twice.

**Analysis of caspase-3 activity.** The Apo-one Homogeneous Caspase-3/7 Assay (Promega, Madison, WI, USA) was used to analyze caspase-3 activity in HCC cells according to the manufacturer's instructions. Further, active caspase-3 was quantified by flow cytometry using the CaspGLOW Fluorescein Active Caspase-3 Staining kit (BioCat, Heidelberg, Germany) according to manufacturer's instructions. Briefly, HCC cells were seeded in 6-well plates (20,000 cells/cm<sup>2</sup>) and incubated with specific concentrations of XN in DMEM containing 10% FCS at 37°C for 24 h. Subsequently, cells were washed two times with PBS, detached by trypsination, resuspended in DMEM supplemented with 10% FCS and incubated with the FITC-conjugated caspase-3 inhibitor (FITC-DEVD-FMK) for 30 min. Thereafter, cells were washed with the provided washing buffer and analyzed by flow cytometry using a Coulter EPICS XL flow cytometer (Beckman Coulter, Krefeld). Percentages of FITC positive (active caspase-3) cells of 10,000 analyzed cells were evaluated with the Expo32 ADC Software version 1.1C (Beckman).

**Analysis of propidium iodide incorporation by flow cytometry.** For determination of viability, cells were incubated with propidium iodide (PI) (Pharmingen, Heidelberg, Germany) and PI incorporation was quantified by flow cytometry as described (15).

**Proliferation assay.** Cell proliferation was measured using the XTT assay (Roche Diagnostic, Mannheim, Germany) according to the manufacturer's instructions as described (16).

**Migration assay.** Migration potential of HCC cells was quantified using the Cultrex 96 Well Cell Migration Assay (Trevigen, Gaithersburg, USA) according to the manufacturer's instructions. Briefly, HCC cells were seeded into the upper compartment of the provided 96-well plate (40,000 cells/well) in DMEM supplemented with XN in specific concentrations as indicated. The lower compartment was filled with DMEM supplemented with conditioned medium from fibroblasts and 10% FCS as chemoattractants. After incubation at 37°C for 5 h cell migration was quantified by fluorimetry with an EMax Microplate Reader (MWG Biotech, Ebersberg, Germany). Further, migration was assessed by Boyden chamber assays as described (17).

**Quantification of activated nuclear NF-κB concentration.** NF-κB was quantified in nuclear extracts with the ELISA-based kit TransAm NF-κB p65 from Active Motif (Rixensart, Belgium) as described (18).

**Expression analysis.** Isolation of total cellular RNA from cultured cells and reverse transcription were performed as described (19). IL-8 mRNA expression was quantified using LightCycler real-time PCR technology (Roche) as described (12) applying the following pair of primers: IL-8 forward: 5'-TCTGCAGCTCTGTGTGAAGGTGCAGTT-3' and IL-8 reverse: 5'-AACCCCTCTGCACCCAGTTTTTCCT-3'.

**Statistical analysis.** Values are presented as mean ± SEM. Comparison between groups was made using the Student's unpaired t-test. Welch's correction was performed when

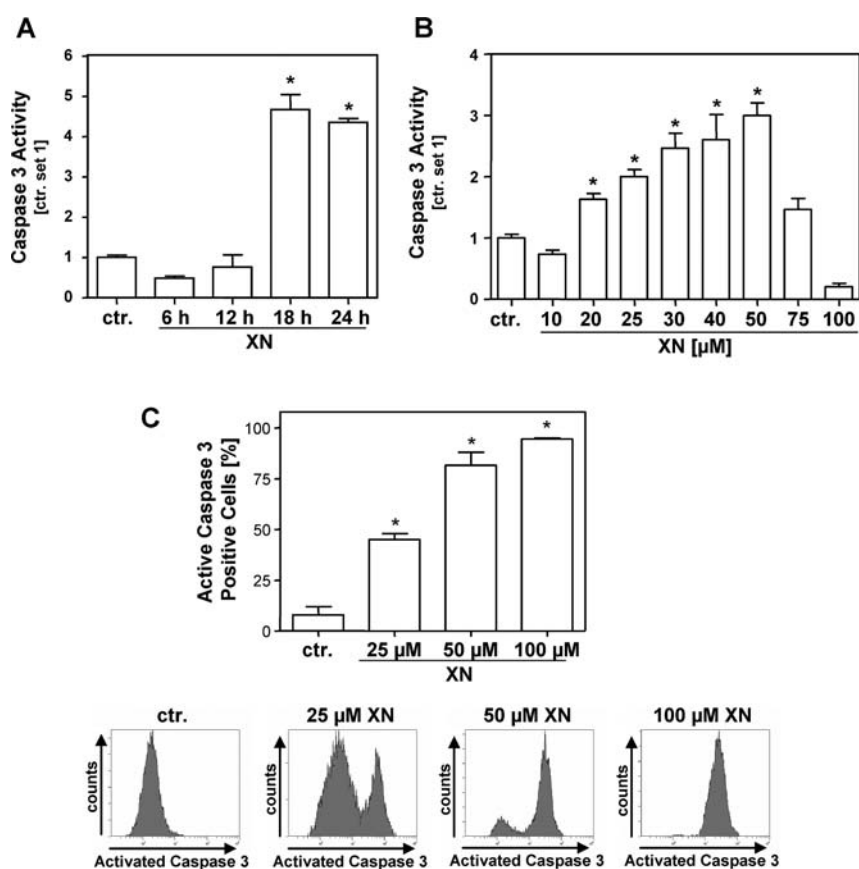


Figure 2. Xanthohumol induced apoptosis in HCC cells. (A) Caspase-3 activity in HepG2 cells stimulated with xanthohumol (XN) at a concentration of 25  $\mu$ M for the time intervals indicated. (B) Caspase-3 activity in Huh7 cells stimulated for 24 h with different XN concentrations as indicated. (C) Percentage of Huh7 cells with active caspase-3 after treatment with XN at indicated concentrations for 24 h determined by flow cytometry. \* $p < 0.05$ , experiments were performed in triplicate and were repeated at least twice.

required. A  $p < 0.05$  was considered statistically significant. All calculations were performed using the statistical computer package GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA).

## Results

*Xanthohumol induces cell death in HCC cells, but not in primary hepatocytes.* First, we analyzed the effects of xanthohumol (XN) on the viability of the two human HCC cell lines HepG2 and Huh7. Microscopic observation revealed a marked decrease of the cell number after incubation with XN at a concentration of 25  $\mu$ M for 24 h. Stimulation with higher XN concentrations led to an almost complete cell detachment (Fig. 1A and B). Consistently, a significant increase of LDH levels was detected in the supernatants of HCC cells after incubation with 25 and 50  $\mu$ M XN (data not shown). In contrast, primary human hepatocytes (PHH) appeared completely unaffected after 24 h incubation with XN concentrations as high as 100  $\mu$ M (Fig. 1C). To confirm this finding, PHH were detached by trypsin and stained with propidium iodide (PI), revealing no significant PI incorporation at any XN concentration up to 100  $\mu$ M (Fig. 1D).

*Xanthohumol induces apoptosis in HCC cells.* To further study the underlying mechanisms leading to HCC cell death upon stimulation with XN we measured the time-dependent

activation of caspase-3 in HepG2 cells incubated with 25  $\mu$ M XN within 24 h. After 12 h incubation caspase-3 activity was unaltered, but 6 h later a significant increase was observed remaining on this elevated level (Fig. 2A). A similar time course of caspase-3 activation induction was observed in Huh7 cells (data not shown).

Next, the effect of different XN concentrations on caspase-3 activity in Huh7 and HepG2 cells were analyzed revealing a concentration dependent increase of caspase-3 activity in both cell lines, reaching a maximum at 50  $\mu$ M XN (Fig. 2B, data for HepG2 not shown). At higher XN concentrations HCC cells detached, and accurate analysis of the caspase-3 was not possible with an ELISA based assay. To bypass this pitfall we additionally performed flow cytometry analysis, applying a FITC-labeled antibody against active caspase-3. Here, basically all Huh7 (Fig. 2C) and HepG2 (data not shown) cells revealed a positive signal for active caspase-3 24 h after stimulation with 100  $\mu$ M XN.

*Xanthohumol inhibits proliferation and migration of HCC cells.* To further characterize the effect of XN on HCC cells, we performed the XTT assay (see Materials and methods) with both cell lines using different XN concentrations. Proliferation was significantly inhibited after incubation with 15  $\mu$ M XN, whereas concentrations higher than 30  $\mu$ M completely abrogated cell proliferation in HepG2 (Fig. 3A) and Huh7 (Fig. 3B) cells.

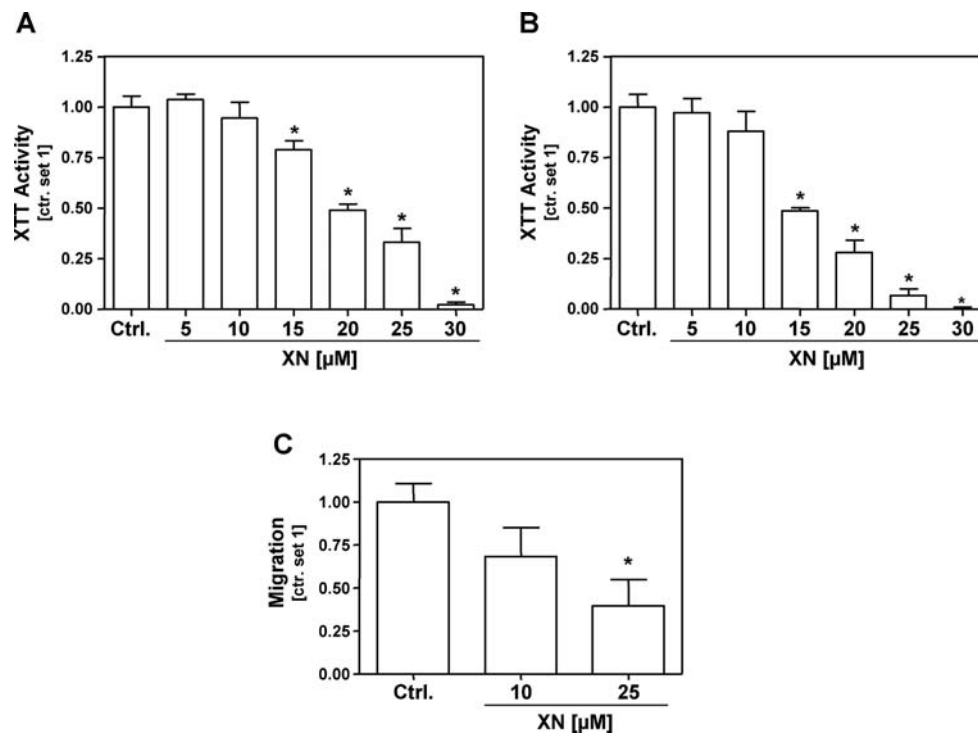


Figure 3. Effect of Xanthohumol on proliferation and migration of HCC cells. Proliferation of (A) HepG2 and (B) Huh7 cells 72 h after stimulation with different concentrations of xanthohumol (XN) relative to control cells (set to 1). (C) Quantification of migration of Huh7 cells incubated with XN (10 or 25  $\mu\text{M}$ ) in comparison to untreated control cells (set to 1). \* $p < 0.05$ , experiments were performed in triplicate and were repeated at least twice.

Next, we analyzed whether XN affects the migration potential of HCC cells *in vitro*. For these experiments the Cultrex cell migration assay (see Materials and methods) was used and a short time span of 5 h was chosen to exclude proapoptotic or anti-proliferative effects of XN in the applied concentrations (10 or 25  $\mu\text{M}$ ). Noteworthy, we observed a significant inhibition of cell migration of Huh7 cells treated with 25  $\mu\text{M}$  XN as compared to untreated control cells (Fig. 3C; similar data of HepG2 cells not shown).

*Xanthohumol inhibits NF- $\kappa$ B activation and IL-8 expression of HCC cells.* XN has been shown to affect NF- $\kappa$ B activity, and we and others have shown that NF- $\kappa$ B plays an important role in hepatocarcinogenesis (17,20,21). Thus, we further analyzed the effect of XN on NF- $\kappa$ B activity in HCC cells. To avoid potential paracrine side effects of dead HCC cells on NF- $\kappa$ B activity we chose a non-toxic XN concentration of 2.5  $\mu\text{M}$  and stimulated the cells for only 3 h. In this case, XN exhibited no effects on basal NF- $\kappa$ B activity in either cell line. However, TNF (10 ng/ml)-induced NF- $\kappa$ B activity was significantly blunted in XN treated Huh7 cells even at this low concentration (Fig. 4A).

Next, we analyzed the effect of XN on IL-8 expression in HCC cells, since IL-8 expression is known to be regulated by NF- $\kappa$ B in HCC cells (22-24), and previous studies indicated that IL-8 is directly or indirectly involved in the progression of HCC (24,25). In accordance with the effects on NF- $\kappa$ B activity, basal IL-8 expression was not affected by stimulation with XN for 24 h, however, TNF induced IL-8 expression was significantly lower in Huh7 cells preincubated with XN as compared to controls (Fig. 4B).

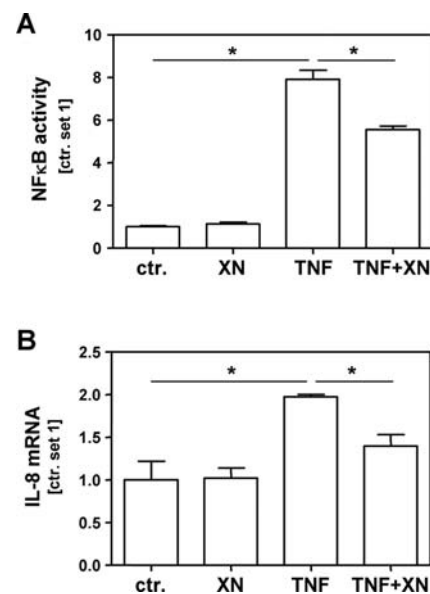


Figure 4. Effect of Xanthohumol on NF- $\kappa$ B activity and IL-8 mRNA expression in HCC cells. (A) Basal and TNF-induced (10 ng/ml, 15 min) NF- $\kappa$ B activity in nuclear extracts of Huh7 cells, which were preincubated with xanthohumol (XN, 2.5  $\mu\text{M}$ , 3 h), compared to control cells. (B) Analysis of basal and TNF (10 ng/ml for 1 h) induced IL-8 mRNA expression by quantitative PCR. \* $p < 0.05$ , experiments were performed in triplicate and were repeated at least twice.

## Discussion

In the present study we aimed to analyze the effects of xanthohumol on viability and tumorigenicity of HCC cells.

We observed significant cell death in HCC cells upon stimulation with XN at a concentration of 25  $\mu\text{M}$ , and analysis of the time course of caspase-3 activation indicated that XN induced cell death is caused by apoptosis rather than other cytotoxic effects at this concentration, while XN concentration higher than 50  $\mu\text{M}$  led to a rapid and almost complete detachment of HCC cells *in vitro*. XN induced cell death has been described in cell lines derived from various cancers at similar concentrations as observed in the present study (1,2,4,26-28). Thus, stimulation of prostate cancer cells with 20  $\mu\text{M}$  XN resulted in an approximately halved viability (4), and XN concentration of 100  $\mu\text{M}$  induced 100% cell death in breast cancer cells (1). Recently, Ho *et al* reported that XN induced apoptosis in two HCC cell lines *in vitro* (29). However, in this study considerably higher XN concentrations (>200  $\mu\text{M}$ ) were required to induce death of all HCC cells. One reason for this discrepancy might be the different HCC cell lines used (HA22T and Hep3B in the study of Ho *et al*, vs. HepG2 and Huh7 cells in our experiments). Furthermore, Ho *et al* used a hop extract containing 95.7% XN for their studies. Thus, confounding antagonizing effects of concomitant hop constituents can not be excluded.

In addition to viability, we analyzed functional effects of XN on HCC cells. Previous reports revealed an anti-proliferative effect of XN on cancerous cells of different origin (2,3,5,30). However, we are the first reporting a growth inhibitory effect of XN on HCC cells at concentrations as low as 15  $\mu\text{M}$ . Most likely the analysis of anti-proliferative effects of XN is overlapped by its pro-apoptotic and cytotoxic effects. However, our dose-response studies clearly indicate that the significant anti-proliferative effect of XN observed already in the low concentration range can not be exclusively explained by these confounding mechanisms of XN action.

Of note, we further revealed that XN inhibits the migratory potential of HCC cells *in vitro*. Also with regard to migration, we chose conditions excluding that the observed effects of XN are only explained by confounding mechanisms on cell viability. To the best of our knowledge, an effect of XN on the migratory potential of cancerous cells has not been described in previous reports.

Furthermore, we demonstrated that XN inhibits TNF induced NF- $\kappa$ B activity in HCC cells *in vitro*, a situation that reflects the *in vivo* situation since HCC almost exclusively develops and progresses, respectively, on the basis of chronic liver inflammation (21). Furthermore, TNF and other members of the TNF superfamily are known to be capable of inducing cell death via death receptor mediated apoptosis pathways in tumor cells, and thus, appear attractive for cancer treatment (31). However, one of the major obstacles regarding their therapeutic application is the adaptive resistance due to activation of the NF- $\kappa$ B pathway (32,33). Therefore, suppression of NF- $\kappa$ B appears as potential approach in overcoming the resistance to TNF induced apoptosis. Moreover, increased NF- $\kappa$ B activation is a common strategy of cancer cells to evade apoptosis, and further, has been shown to promote proliferation as well as migration of tumorous cells including HCC cells (17,34-37). In addition, NF- $\kappa$ B regulates the expression of the pro-inflammatory chemokine IL-8 in HCC cells (23,38), and previous studies have shown that IL-8 promotes the progression of HCC (24,25).

Together, these data indicate the potential of XN as a therapeutic agent for HCC treatment. However, very few studies have addressed the important question whether XN exhibits (unwanted) effects on healthy tissue or non-tumorous cells, respectively. Yang *et al* described loss of viability in approximately 90% of 3T3-L1 cells, a murine adipocyte cell line, after 24 h treatment with 75  $\mu\text{M}$  XN (39). In the (non-malignant) murine hepatoma cell line AML12 the maximal applied concentration of 225  $\mu\text{M}$  XN (24 h) induced toxicity in approximately 50% of the cells as reported by Ho *et al* (29). However, to the best of our knowledge, no data have been reported concerning the effect of XN on primary human cells. In the present study we analyzed the effect of XN on the viability of primary human hepatocytes. Noteworthy, we found that viability of PHH was not affected after 24 h stimulation with a XN concentration as high as 100  $\mu\text{M}$ . This is four-to ten-fold higher in comparison to the XN concentrations affecting viability and function of HCC cells under exactly the same experimental conditions.

These data further promote the use of XN as anti-cancer therapeutic, and first insight regarding bioavailability and metabolism of flavonoids and polyphenols suggest that particularly HCC appears as predestined cancer for oral XN treatment. Metabolism of flavonoids is rather complex and depends on the structure, the dose and the matrix, but there is evidence that several congeners reach (to a certain extend) the small intestine unchanged (40), where absorption into the mesenteric circulation takes place. Accordingly, it can be presumed that upon oral administration of XN hepatocytes as well as HCC cells are exposed to intact XN reaching the liver via portal circulation after absorption. In general, metabolism of most xenobiotics takes place in hepatocytes, and furthermore, it has been already shown that XN is effectively metabolized by rat and human liver microsomes *in vitro* (41-43). These studies suggest that XN is probably completely metabolized in the liver, and therefore, it may be predicted that circulating plasma concentrations will not reach potentially effective levels for most other cancers if XN is administered orally. This phenomenon is well-known for many other polyphenolic compounds (44).

*In vivo* studies in mice and rats (45,46) as well as our *in vitro* experiments using human hepatocytes indicate a very good safety profile of XN. Still, further safety and efficacy studies are required to evaluate the suitability of XN as a therapeutic agent for HCC. However, the demonstrated pro-apoptotic, anti-proliferative, anti-migratory and anti-inflammatory effects of XN on HCC cells *in vitro* may act synergistically also *in vivo*, and herewith, XN appears as attractive and promising therapeutic agent for this highly aggressive tumor.

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authors with Joh. Barth & Sohn GmbH are as follows: C.H. is a consultant, and C.D. is working in the laboratory of C.H.

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