

Anti-proliferative and apoptosis-inducing properties of Xanthohumol, a prenylated chalcone from hops (*Humulus lupulus* L.)

Julia Strathmann[§] & Clarissa Gerhauser

Division Epigenomics and Cancer Risk Factors, German Cancer Research Center, Heidelberg, Germany

[§]Current address: German Center for Neurodegenerative Diseases (DZNE) - Munich & Adolf-Butenandt-Institute, Department of Biochemistry, Munich, Germany

Anti-proliferative and apoptosis-inducing properties of Xanthohumol, a prenylated chalcone from hops (<i>Humulus lupulus</i> L.)	1
Abstract.....	2
1 Introduction	3
2 Xanthohumol as a cancer chemopreventive lead structure	3
3 Cell-growth inhibitory potential of Xanthohumol	4
4 Mechanisms of apoptosis induction by Xanthohumol	5
4.1 Death receptor pathway, TNF and NF-κB	5
4.2 Mitochondria-mediated apoptosis induction	8
4.3 The role of reactive oxygen species (ROS).....	9
4.4 ER stress and unfolded protein response.....	11
5 Inhibition of tumor growth <i>in vivo</i>	14
6 Summary and Conclusion.....	15
7 References	16
Annex (Table 1).....	21

Abstract

Xanthohumol (XN)* is a prenylated chalcone found at high concentrations in hop cones (*Humulus lupulus* L.). XN has been characterized as a promising cancer chemopreventive lead structure that acts *via* a broad spectrum of bioactivities. This chapter summarizes the anti-proliferative and apoptosis-inducing potential of XN and gives a detailed overview of underlying mechanisms and pathways targeted by XN to induced programmed cell death. XN is a potent inhibitor of NF- κ B and inhibits activation of the death-receptor pathway by tumor necrosis factor (TNF). In various cell lines, XN treatment results in an immediate transient increase in mitochondria-derived reactive oxygen species (ROS) that is considered as the initial trigger of apoptosis induction *via* the intrinsic pathway by breakdown of the mitochondrial membrane potential, release of cytochrome *c* and activation of the caspase cascade. Oxidative stress may also contribute to the activation of endoplasmatic reticulum (ER) stress and unfolded protein response recently identified as a novel mechanisms underlying XN-mediated apoptosis induction.

*Abbreviations:

AML, acute myelocytic leukemia; A-SMase, acid sphingomyelinase; ATF, activating transcription factor; ATP, adenosine triphosphate; BiP, immunoglobulin-heavy-chain binding protein; BPH, benign prostate hyperplasia; CHOP, CAAT/enhancer-binding protein (C/EBP) homologous protein; CLL, chronic lymphocytic leukemia; Cox, cyclooxygenase; CYP, cytochrome P450; DC, dendritic cells; DCF-DA, dichlorofluorescein-diacetate; DHE, dihydroethidium; DISC, death-inducing signaling complex; DMBA, dimethylbenz-*[a]*-anthracene; DMSO, dimethylsulfoxide; DR, death receptor; ER, endoplasmatic reticulum; FADD, Fas-associated death domain; FITC, fluorescein isothiocyanate; Gadd153, growth arrest and DNA damage 153; GRP78, glucose-regulated protein 78; GSH, glutathione; H₂O₂, hydrogen peroxide; HUVEC, human umbilical vein endothelial cells; IC₅₀, half-maximal inhibitory concentration; IKK, I- κ B kinase; IL, interleukin; Ire1 α , inositol-requiring 1 α ; LDH, lactate dehydrogenase; MMP, matrix metalloprotease; MnTMPyP, manganese(III) tetrakis(1-methyl-4-pyridyl)porphyrin; MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazoliumbromide; NAC, N-acetyl cysteine; NAD(P)H, nicotinamide adenine dinucleotide phosphate; NF- κ B, nuclear factor κ B; O₂⁻, superoxide anion radicals; OH[•], hydroxyl radicals; PARP, poly(ADP-ribose)polymerase; PERK, double stranded RNA-dependent protein kinase (PKR)-like ER kinase; RIP, receptor interacting kinase; ROS, reactive oxygen species; SCID, severe combined immuno-deficient; SM, sphingomyelin; SOD, superoxide dismutase; SRB, sulforhodamine B; TNF, tumor necrosis factor; TNF-R1, TNF-receptor 1; TRAF, TNF receptor-associated factor; TRAIL, TNF-related apoptosis-inducing ligand; Trb3, Tribble homolog 3; TUNEL, TdT-mediated dUTP-biotin nick end labeling; UPR, unfolded protein response; XBPI, X-box-binding protein 1; XN, Xanthohumol; Ψ_m , mitochondrial membrane potential; ρ^0 , rho zero

1 Introduction

Evading apoptosis has been recognized as one of the hallmarks of cancer cells [1]. Consequently, the induction of apoptosis by cancer chemotherapeutic or chemopreventive agents is one of the key mechanisms to effectively kill cancer cells and thus prevent or inhibit tumor growth. Anti-proliferative action has been demonstrated for a large number of natural compounds in human cancer cell lines as well as in *in vivo* models of carcinogenesis. One of these natural compounds is xanthohumol (XN, 2',4,4'-trihydroxy-3'-prenyl-6'-methoxychalcone, Fig. 1), a prenylated chalcone found in hops (*Humulus lupulus* L.).

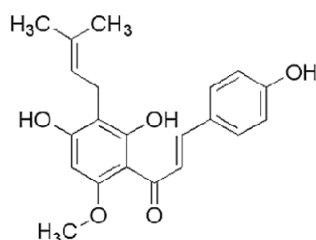


Fig. 1 Chemical structure of xanthohumol (2',4,4'-trihydroxy-3'-prenyl-6'-methoxychalcone)

Prenylated hop flavonoids are secreted together with bitter acids and essential oils by lupulin glands of the female hops inflorescences (hop cones). Since lupulin glands do not express the enzyme necessary for the conversion of chalcones to flavanones, they exclusively produce chalcone type flavonoids with XN as the most abundant one (82-89% of the total amount of prenylated flavonoids in European hop varieties) [2].

2 Xanthohumol as a cancer chemopreventive lead structure

Interest in the cancer chemopreventive potential of hops components started in the late 1990s, when prenylated flavonoids from hops were first described to modulate carcinogen metabolism *in vitro* [3,4] and to possess antioxidant [5], anti-proliferative and cytotoxic activity [6]. Subsequently, we identified XN as a broad spectrum cancer chemopreventive agent able to interfere with the initiation, promotion and progression phase of carcinogenesis [7]. Several recent reviews have comprehensively summarized the cancer preventive and health promoting activities of XN and other hops components [7-14]. Therefore, we will present here only a short overview of XN-mediated chemopreventive activities to emphasize that anti-proliferative and apoptosis-inducing properties, although the focus of this chapter, are not the sole or most important biological activities of this interesting lead structure.

During the initiation step of carcinogenesis, XN modulates xenobiotic metabolism by inhibiting the phase I enzyme cytochrome P450 (CYP) 1A and monofunctionally inducing phase II enzymes, such as NAD(P)H: quinone oxidoreductase and intracellular glutathione levels (GSH), leading to reduced metabolic activation and increased detoxification of xenobiotics and carcinogens [3,7]. Antioxidant and radical scavenging properties can also contribute to the inhibition of tumor initiation by XN [7] (reviewed in [9,13]). XN inhibits cyclooxygenases (Cox)-1 and -2 activities as well as the production of nitric oxide [7]. These anti-inflammatory properties may contribute to the inhibition of tumor promotion. XN also acts as an anti-inflammatory agent by inhibition of nuclear factor κ B (NF- κ B) signaling and subsequent downregulation of pro-inflammatory key factors [15-18]. Estrogen-mediated tumor promotion may be prevented by anti-estrogenic effects of XN that were demonstrated *in vitro* by the inhibition of estrogen-mediated alkaline phosphatase activation in human endometrial cancer cells [7,19]. XN also inhibits the enzyme aromatase (CYP19), which plays a crucial role in the conversion of testosterone to estrogen [13,20]. In the progression phase, XN affects cell proliferation by induction of cell differentiation [7] and apoptosis (as outlined below). In addition, XN may inhibit tumor progression by inhibition of angiogenesis. This was demonstrated in a human *in vitro* anti-angiogenesis model using fragments of human placenta, by downregulation of pro-angiogenic signaling, and by inhibition of endothelial cell migration and vessel formation using human microendothelial cells [9]. *In vivo*, inhibition of angiogenesis was demonstrated in human breast cancer xenografts in a skinfold chamber model [21], and with a matrigel sponge angiogenesis assay as described by Albini *et al.* [15]. These activities contribute to the inhibitory effects of XN during malignant progression of tumorigenesis. Breast cancer chemopreventive potential of XN was first indicated by inhibition of 7,12-dimethylbenz-[a]-anthracene (DMBA)-induced preneoplastic lesions in a mammary mouse organ culture model at low nM concentrations [7]. Recently, we have demonstrated that XN also possess breast cancer preventive efficacy in the DMBA-induced rat mammary carcinogenesis model. Application of XN at a dose of 100 mg/kg bodyweight/day significantly inhibited tumor latency, tumor multiplicity ($p < 0.05$) and tumor weight ($p = 0.07$) when applied during the initiation and promotion phase of carcinogenesis (Strathmann *et al.*, in preparation).

3 Cell-growth inhibitory potential of Xanthohumol

The first indication of anti-proliferative potential of XN was reported by Miranda *et al.* in 1999 [6]. Since then, more than 25 studies have investigated XN in anti-proliferation and cytotoxicity assays using ovarian, breast, endometrial, cervical, prostate, colon, liver, and lung cancer, as well as leukemia, myeloma, sarcoma and melanoma cell lines, macrophages, adipocytes, dendritic cells and T-cells (summary in Table 1 in the Annex). Early studies investigated anti-proliferative activity based on [3 H]-thymidine incorporation, sulforhodamin B (SRB) or crystal violet

staining, lactate dehydrogenase (LDH) release, MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazoliumbromide) reduction, calcein hydrolysis, trypan blue exclusion and cell counting, and determined halfmaximal inhibitory concentrations (IC₅₀ values) of cell viability in the range of 0.5 to 25 μM, depending on cell line and incubation times. Cell growth inhibition by XN is apparently not cancer site or organ specific, at least not in *in vitro* studies. Interestingly however, XN more potently reduced cell growth of HT-1080 sarcoma cell under hypoxic conditions than under normoxic conditions [22]. Also, data by Monteiro *et al.* suggest that XN-mediated inhibition of breast cancer cell growth may be partly related to a reduction of estrogen levels by aromatase inhibition [23]. Importantly, primary hepatocytes were more resistant to the anti-proliferative effects of XN than liver cancer cells [24-26].

4 Mechanisms of apoptosis induction by Xanthohumol

Generally, apoptosis can be induced by two major pathways: the extrinsic, death receptor-mediated and the intrinsic, mitochondria-mediated pathway [27]. In addition, apoptosis can be triggered by endoplasmatic reticulum (ER) stress and unfolded protein response [28,29]. There is accumulating evidence that XN targets all three pathways.

4.1 Death receptor pathway, TNF and NF-κB

Death receptors of the TNF (tumor-necrosis factor) receptor superfamily such as TNF receptor 1 (TNF-R1), CD95 (APO-1/Fas), and TNF-related apoptosis-inducing ligand (TRAIL) receptors DR4 and DR5 play an important role in the extrinsic pathway of apoptosis induction. As an example, activation of the CD95/Fas by the CD95/Fas ligand results in receptor aggregation and recruitment of the adaptor molecule FADD (Fas-Associated Death Domain) and initiator caspase-8 (Fig. 2, right). Consequently, caspase-8 becomes activated and initiates apoptosis by cleavage of downstream effector caspases. Caspases are synthesized as inactive pro-caspases and act in a caspase cascade to initiate apoptosis. Initiator caspases are characterized by longer pro-domains that mediate the assembly of activating complexes, such as the death-inducing signalling complex (DISC), resulting in the transduction of death signals. The major effector caspases-3, -6 and -7 execute apoptosis by cleavage of key cellular proteins that cause the typical morphological changes observed in cells undergoing apoptosis, including apoptotic and structural proteins, cell cycle proteins and proteins belonging to the cellular DNA repair machinery (summary in [27]). Cleavage of the DNA repair-associated enzyme

poly(ADP-ribose)polymerase (PARP) is accepted as a prominent marker of apoptosis.

Activation of sphingomyelinases such as the acid sphingomyelinase (A-SMase) results in the formation of ceramide from sphingomyelin (SM). Ceramide is acting as a second messenger involved in regulating various cellular functions including proliferation and apoptosis [30]. Activation of death receptors has been shown to activate A-SMase, enhancing ceramide production, which in turn facilitates death receptor clustering, DISC formation and caspase-8 activation [31].

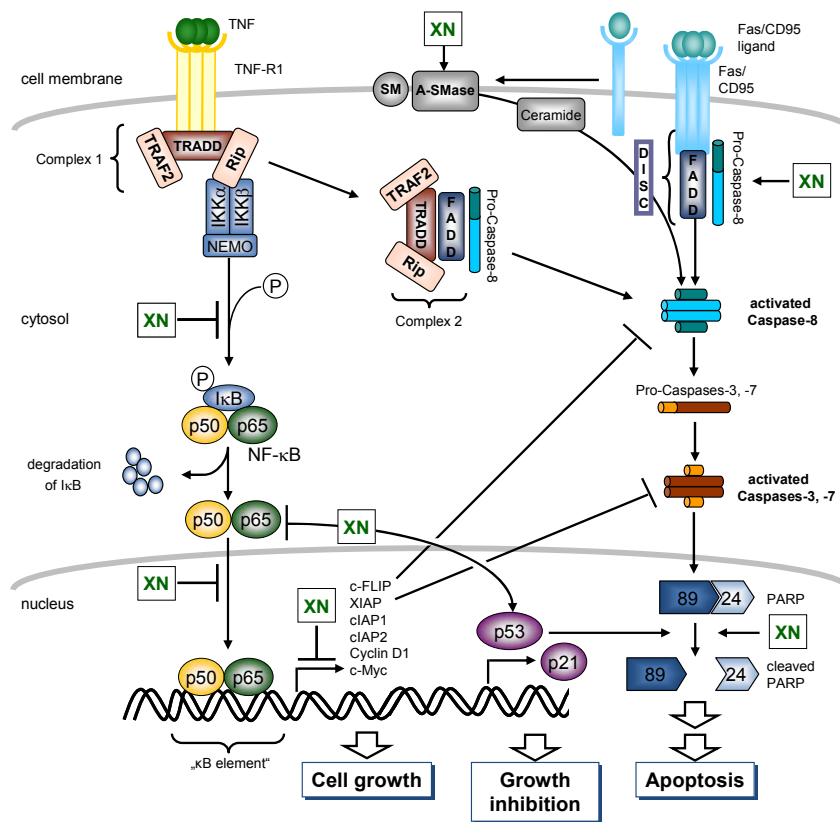


Fig. 2 Induction of apoptosis via the death receptor pathway, and the influence of XN on these mechanisms (see text for description)

TNF is a multifunctional pro-inflammatory protein that activates the NF-κB pathway through binding to TNF-receptors (Fig. 2, left). After TNF-binding, the adaptor protein TRADD is recruited to the activated TNF-R1 and serves as a platform for binding of TNF receptor-associated factor (TRAF) 2 and the receptor interacting kinase (RIP), forming complex 1. TNF activates NF-κB through phosphoryla-

tion and subsequent ubiquitin-mediated degradation of its inhibitor I- κ B by I- κ B kinase (IKK), which is recruited to the TNF-R1 complex through TRAF2 and stabilized by RIP. Degradation of I- κ B liberates NF- κ B and allows nuclear translocation, where it interacts with the κ B element. In a second step after formation of the TNF-R1 complex 1, TRAF2 and RIP interact with FADD and pro-caspase 8, forming complex 2 that results in activation of caspase-8 and the caspase cascade.

Different from Fas and TRAIL signalling, TNF does not induce apoptosis spontaneously, since activation of NF- κ B mediates a strong pro-survival mechanism. NF- κ B is involved in the regulation of gene expression of about 200 genes involved in inflammation, development, cell growth, and inhibition of apoptosis [32]. Consequently, the cell-death inducing capacity of TNF is only apparent when NF- κ B activation is blocked. Ak and Levine have recently postulated that NF- κ B and p53 have mutually exclusive functions: inactivation of NF- κ B enhances the stability of p53, which then contributes to cell growth inhibition and apoptosis induction [32].

In 2005, we were first to describe that XN induced apoptosis in the HCT-116 derived colon cancer cell line 40-16 *via* the extrinsic apoptotic pathway [33]. XN treatment led to cleavage and activation of the initiator caspase-8 in a dose- and time-dependent manner. Consequently, XN also activated downstream effector caspases-3 and -7 and PARP cleavage as a marker of apoptosis induction. Several other groups subsequently demonstrated similar activation of the caspase cascade, PARP cleavage and apoptosis induction by XN in breast cancer cell lines and patient-derived B-cell lymphocytic leukemia (B-CLL) cells [34,35] (Table 1). Interestingly, Xuan *et al.* recently identified a role of A-SMase-derived ceramide in XN-mediated apoptosis induction in dendritic cells (DC) [36]. Unlike in DC from wildtype mice, in DC from A-SMase knockout mice, XN was unable to activate caspase-8 and -3 and to stimulate PARP cleavage and DNA fragmentation, underlining the importance of A-SMase activity for XN-mediated apoptosis induction in DC.

An influence of XN on NF- κ B signaling was first described by Albini *et al.* in 2006. The authors investigated anti-angiogenic modes of action of XN. Treatment of human umbilical vein endothelial cells (HUVEC) with TNF for 15 min resulted in nuclear translocation of NF- κ B, which was completely blocked by pretreatment with 10 μ M XN. This was attributed to the inhibition I- κ B phosphorylation by XN. In addition to blocking TNF-induced NF- κ B activation, XN inhibited the constitutive activity of NF- κ B in BPH-1 prostate epithelial cells, but not in the PC-3 prostate cancer cell line. Effects in BPH-1 cells were not associated with inhibition of NF- κ B nuclear translocation. Still, caspases were activated, and XN treatment resulted in cell growth inhibition and cell death [16]. In MM6 and U937 leukemia cells stimulated with TNF, inhibition of NF- κ B activation by XN led to downregulation of matrix metalloproteases (MMP) expression and reduced invasive potential. Also, XN reduced proliferation of MM6 and U937 leukemia cells and primary samples from acute myelocytic leukemia (AML) patients [17]. Anti-

leukemic efficacy of XN was further confirmed in the myeloid leukemia cell line K562 positive for the tyrosine kinase Bcr-Abl. Bcr-Abl activates several signaling pathways including Akt and NF- κ B. XN treatment reduced cell viability and led to apoptosis induction. Also, cell invasion was reduced. Both effects were attributed to suppression of NF- κ B activation as well as I κ B and IKK expression. Levels of the anti-apoptotic protein survivin, which is induced by Bcr-Abl, and of Bcr-Abl itself were reduced by XN, whereas expression of p53 and its effector p21 were elevated [37]. Most of these XN-mediated effects on cell proliferation, apoptosis induction, Bcr-Abl expression, and NF- κ B activation were mediated by increased oxidative stress after XN treatment, and were prevented by pretreatment with the antioxidant N-acetyl cysteine (NAC)(see also below). In a more mechanistic study in leukemia cell lines, Harikumar *et al.* confirmed that XN induced apoptosis by affecting NF- κ B signaling. XN treatment blocked constitutive NF- κ B activity and NF- κ B activation by TNF, prevented nuclear translocation of the NF- κ B p65 subunit to the nucleus, suppressed NF- κ B-regulated proliferative (cyclin D1, c-Myc) as well as anti-apoptotic gene products (Bcl-xL, XIAP, cIAP1, cIAP2), thus promoting apoptosis. The authors postulate that XN may directly interact with cysteine residues of I κ B kinase (IKK) and the p65 subunit of NF- κ B through its unsaturated ketone moiety [18]. Inhibition of NF- κ B activation by XN and increased caspases 3 activity was also involved in apoptosis induction in hepatic stellate cells and Huh7 human liver cancer cells [25,26]. In a recent study, Szliszka *et al.* demonstrated that combined treatment with TRAIL and XN or a series of other chalcones enhanced the apoptosis inducing capacity of TRAIL in prostate cancer cells [38].

4.2 Mitochondria-mediated apoptosis induction

The intrinsic mitochondrial pathway of apoptosis induction is initiated by Bcl-2 family proteins, which regulate the passage of small molecules like cytochrome *c* through the mitochondrial permeability transition pore (Fig. 3). The Bcl-2 protein family includes anti-apoptotic (e.g. Bcl-2, Bcl-xL, Bcl-w, Mcl-1) and pro-apoptotic proteins (e.g. Bax, Bak, Bad). The small BH3 domain-only protein Bid activates pro-apoptotic Bax after cleavage by caspase-8 and thus interconnects the death receptor- and the mitochondrial pathway. Intracellular stress signals trigger the translocation of Bax from the cytosol to the mitochondria, where it homodimerizes and contributes to the permeabilization of the mitochondrial membrane. Release of cytochrome *c* then activates assembly of the multiprotein complex Apoptosome, resulting in activation of pro-caspase-9 and the downstream effector caspase cascade [27].

Our study with 4016 colon cancer cells indicated that XN not only activates the death receptor pathway, but also the intrinsic apoptosis pathway [33]. XN decreased the expression of anti-apoptotic Bcl-2, leading to cleavage and subsequent

activation of pro-caspase-9 and downstream effector caspases. Further, XN treatment induced p53 and pro-apoptotic Bax expression in BPH-1 prostate epithelial cells, whereas anti-apoptotic Bcl-2 was downregulated [16]. Lust *et al.* demonstrated induction of apoptosis by XN *via* the mitochondrial pathway in human chronic lymphocytic leukemia (CLL) cells. XN reduced the expression of Bcl-2, Mcl-1 and Bid, and induced caspase-9 and -3 activity [39].

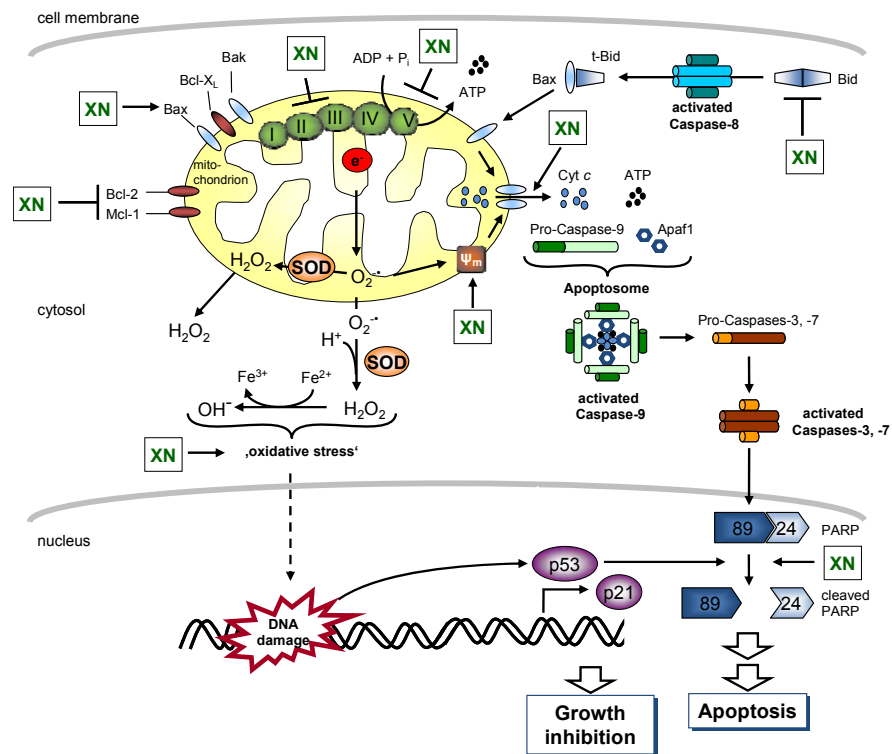


Fig. 3 Induction of apoptosis *via* the mitochondrial pathway, including the role of reactive oxygen species (ROS). Mechanisms targeted by XN are indicated (see text for description)

4.3 The role of reactive oxygen species (ROS)

Recently, ROS have been identified as key redox regulators of cellular signaling cascades, metabolic processes and transcription factors. Numerous cellular pathways generate ROS, with hydrogen peroxide (H₂O₂), superoxide anion radicals (O₂^{•-}) and hydroxyl radicals (OH[•]) being the most abundant ones [40]. In aerobic

cells, mitochondria are believed to be the major source of ROS, since roughly 1-2% of all transported electrons escape oxidative phosphorylation and reduce O_2 to $O_2^{\cdot-}$ [41-44].

Acute high levels of ROS oxidize intracellular proteins, inactivate iron-sulfur complex-containing enzymes and damage cellular compartments, which ultimately trigger cells into apoptosis. For example, mitochondrial dysfunction has been shown to play a key role in the induction of apoptosis [45,46]. Impaired mitochondrial functions suppress mitochondrial metabolism, imbalance the mitochondrial membrane potential, block respiration and oxidative phosphorylation [47], finally leading to apoptosis induction [48]. Interestingly, cells with an imbalanced redox homeostasis are apparently more susceptible to oxidative stress-induced apoptosis than normal cells. Therefore, induction of ROS might be very effective in eliminating cancer cells by disrupting mitochondrial functions and inducing apoptosis [49].

Despite its antioxidant activity [7], several recent reports suggest that induction of apoptosis by XN is linked to the induction of ROS (Fig. 3). As a first indication, Yang *et al.* reported in 2007 that XN inhibited adipocyte differentiation and subsequently induced apoptosis in human preadipocytes *via* a ROS-mediated mechanism. Incubation of preadipocytes with XN resulted in a transient increase in 'oxidative stress', detected by dichlorofluorescein (DCF) fluorescence. This was accompanied by a rapid breakdown of the mitochondrial membrane potential and release of cytochrome *c* from the mitochondria to the cytosol, activation of caspase-3 and -7, PARP cleavage and apoptosis induction indicated by single stranded DNA. The role of ROS induction was confirmed by pre-treatment with the antioxidants ascorbic acid and β -mercaptoethanol, which efficiently prevented XN-mediated apoptosis induction [50]. As mentioned above, data by Monteghirfo *et al.* also suggested that apoptosis induction by XN in K562 leukemia cells was related to the induction of 'oxidative stress' detected with the fluorescent dye DCF diacetate (DA). XN-mediated effects, *i.e.* inhibition of NF- κ B activation, reduced Bcr-Abl expression, inhibition of cell proliferation, and apoptosis induction were inhibited by co-exposure with the antioxidant NAC. The authors speculated that increased ROS levels might lead to DNA damage, which could activate p53 to establish an amplification loop [37].

Only recently, we could provide detailed information on the nature and source of ROS induced after XN treatment, and their link to apoptosis induction [51]. We confirmed a rapid time- and dose-dependent increase in intracellular ROS formation detected by DCF-DA fluorescence in BPH-1 prostate epithelial cells by XN. DCF-DA is considered as a sensor for unspecific 'oxidative stress' rather than for a particular ROS [52]. By using the dye dihydroethidium (DHE), which is specifically oxidized by $O_2^{\cdot-}$ [53], we could demonstrate that XN treatment resulted in enhanced $O_2^{\cdot-}$ -generation. $O_2^{\cdot-}$ induction by XN was transient and significantly scavenged by co-treatment with the antioxidants ascorbic acid and NAC, as well as by pre-treatment with the superoxide dismutase (SOD) mimetic MnTMPyP

(manganese(III) tetrakis(1-methyl-4-pyridyl)porphyrin). In BPH-1 cells loaded with MitoSOX Red specific for mitochondrial $O_2^{\cdot-}$, XN treatment caused a rapid increase in red fluorescent staining of mitochondria, suggesting that mitochondria are targeted by XN, leading to $O_2^{\cdot-}$ -formation (Fig. 4). In contrast, XN treatment of BPH-1 ρ^0 (rho zero) cells (characterized by non-functional mitochondria) in the presence of DHE resulted in significantly lower DHE oxidation than in intact BPH-1 cells, further confirming the important role of mitochondria in $O_2^{\cdot-}$ -generation induced by XN.

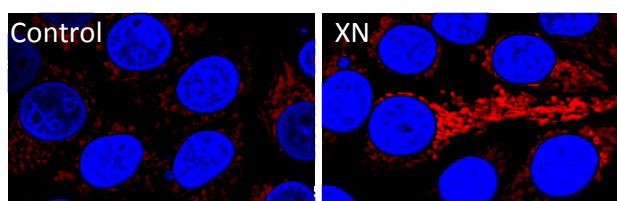


Fig. 4 Mitochondria are the source of XN-induced $O_2^{\cdot-}$. MCF-7 cells were loaded with MitoSOX Red and then incubated with 50 μ M XN for 25min. Red fluorescent staining of mitochondria indicates $O_2^{\cdot-}$ formation, blue staining depicts the position of nuclei. Pictures were acquired by confocal microscopy.

Blocking of mitochondrial respiration is believed to be one of the major sources of mitochondrial $O_2^{\cdot-}$ formation. When oxygen consumption was measured in isolated mouse liver mitochondria, XN treatment was as potent in blocking mitochondrial respiration as Antimycin A, a prominent inhibitor of Complex III of the respiratory chain. A more detailed analysis of XN-mediated effects on complexes of the respiratory chain indicated that XN nonspecifically inhibited Complexes I to III at high concentrations, blocked the electron flux from Complex I to Complexes II and III and caused a rapid depletion of ATP. These data supported the hypothesis that XN inhibits mitochondrial respiration. Consistently, XN caused a rapid breakdown of the mitochondrial membrane potential (Ψ_m), followed by the release of cytochrome *c* to the cytosol and induction of PARP cleavage in a time- and dose-dependent manner. Importantly, anti-proliferative as well as apoptosis-inducing effects of XN were significantly reduced by co-treatment with the SOD mimetic MnTMPyP. Overall, these data strongly suggest that XN-mediated $O_2^{\cdot-}$ formation is the initial trigger of XN-induced apoptosis [51].

4.4 ER stress and unfolded protein response

Besides the death receptor- and the mitochondria-mediated pathway of apoptosis induction, endoplasmic reticulum (ER) stress and unfolded protein response (UPR) is another cellular program activating apoptosis cascades. Interestingly, ER stress is closely linked to oxidative stress. Protein folding itself is an oxidizing

process that leads to the generation of ROS during oxidizing protein folding and the formation of disulfide bonds [54]. ROS can target ER-based calcium channels, stimulating the release of Ca^{2+} ions to the cytosol, which then accumulate in the inner matrix of mitochondria, disrupt the electron transport chain and stimulate the production of more ROS. Excessive ROS production and changes in cellular redox status then directly or indirectly affect protein folding and aggravate ER stress (Fig. 5).

The ER is the cellular site of protein biosynthesis, folding, assembly and modifications. It is composed of protein chaperones, proteins that catalyze folding and sensors for the detection of mis- or unfolded proteins. Also, it is a major calcium store and functions as a sensor to signals mediated by growth factors, hormones, changes in energy levels, nutrient availability and redox status [55]. Alterations in cellular homeostasis that cause accumulation of unfolded proteins in the ER lumen by an imbalance between protein folding demand and protein folding capacity (referred to as ER stress) activate the unfolded protein response (UPR).

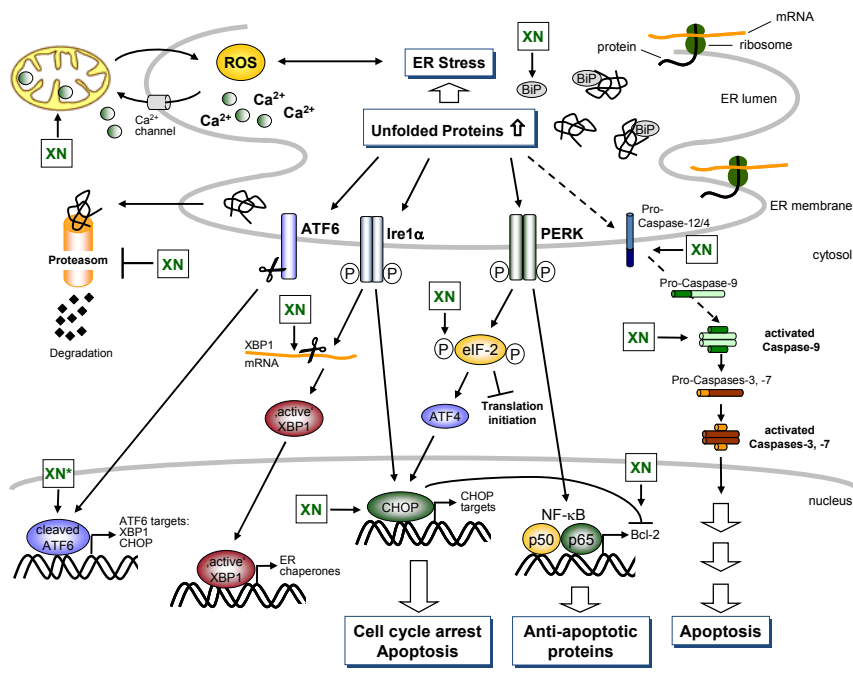


Fig. 5 Link between ER stress and unfolded protein response (UPR) and induction of apoptosis, adapted from [28] and [55]. Mechanisms targeted by XN in CLL cells are indicated (from [39], *only in T47D cells used as control)

The UPR signaling cascades are activated by three ER membrane-localized sensor proteins: ATF6 (activating transcription factor 6), Ire1 α (inositol-requiring 1 α) and PERK (double stranded RNA-dependent protein kinase (PKR)-like ER kinase). Under non-stressed conditions, they are maintained in an inactive state through interaction of their luminal unfolded protein-sensing domain with the abundant ER chaperone BiP (immunoglobulin-heavy-chain binding protein, also known as glucose-regulated protein GRP78). ER stress causes release of BiP and activates PERK through homodimerization and *trans*-autophosphorylation. Activated PERK then phosphorylates the translation initiation factor eIF2 α , thereby reducing the general frequency of mRNA translation initiation. On the other hand, specific mRNAs such as the basic leucine zipper domain (bZIP)-containing transcription factor ATF4 are preferentially translated and activate the transcription of UPR target genes involved in amino acid biosynthesis, antioxidative stress response and ER stress-induced apoptosis. PERK is also required for NF- κ B activation which mainly up-regulates anti-apoptotic proteins (see above) and thus contributes to the balance between survival and cell death. In addition to PERK, ER stress activates autophosphorylation of Ire1 α , which has protein kinase and site-specific endoribonuclease (RNase) activity. It initiates the removal of a 26 base intron from the mRNA of XBP1 (X-box-binding protein 1), resulting in the translation of 'active' XBP1 with potent transcription factor activity [55]. As a third important factor, ER stress initiates the processing of ATF6 to release a functional fragment ('cleaved' ATF6) that acts as a transcription factor with similar functions as 'active' XBP1. These transcription factors stimulate the transcription of UPR target genes including ER chaperones and enzymes that promote protein folding, maturation, secretion and ER-associated protein degradation [55].

If the protein-folding defect is persisting and cannot be resolved, the UPR will initiate apoptosis to remove stressed cells. Apoptosis induction *via* ER-stress is mainly mediated by the bZIP transcription factor CHOP (CAAT/enhancer-binding protein (C/EBP) homologous protein, also known as Gadd153 (growth arrest and DNA damage 153)). For maximal induction of CHOP, activation of all three ER-stress signalling pathways is required. Activation of CHOP target genes such as Gadd34, Trb3 (tribble homolog 3) and DR5 (death receptor 5) induces growth arrest and apoptosis. CHOP has also been implicated in repressing the transcription of anti-apoptotic Bcl-2. In addition to CHOP, pro-caspase-12 (probably represented by pro-caspase-4 in humans) associated with the ER membrane is involved in the induction of apoptosis during ER stress. The mechanisms of caspase 12 activation by ER stress are presently not clear. However, it activates processing and activation of caspase-9 and the downstream caspase cascade [28].

Lust *et al.* have only recently provided first indication that XN induces apoptosis *via* an UPR-mediated mechanisms in CLL cells [39]. The authors first demonstrated activation of caspases-3, -4, and -9 after XN treatment of CLL cells for 12h and more prominently for 24h. Next, they detected upregulation of the chaperone BiP (GRP78) at the mRNA and protein level. XN activated the ER stress sensor IRE1 α , indicated by detection of the processed, shorter XBP1 mRNA.

However, active XBP1 protein was not detectable by Western blotting. The authors concluded that either CLL cells do not translate the processed XPB1 transcript, or that levels were too low for detection by Western blotting. Cleaved ATF6 was also not unambiguously detectable by Western blotting in CLL cells, whereas XN treatment clearly induced the processing of ATF6 in T47D breast cancer cells used as a positive control. PERK activation was demonstrated by eIF2 α phosphorylation, which was strongly enhanced by XN treatment already after 3h of treatment. Also, CHOP protein levels were strongly induced by XN treatment for 12 and 24h. The authors detected ROS by DCF-DA fluorescence microscopy. However, since the influence of co-treatment with antioxidants was not tested, it is unclear whether ROS induction is a cause or consequence of UPR. XN also inhibited protein degradation *via* the proteasome and caused an accumulation of ubiquitinated proteins. These data provide an interesting new insight into the apoptosis inducing capacity of XN in CLL cells. Further studies have to demonstrate whether these observations are also relevant for other cell types and *in vivo*.

5 Inhibition of tumor growth *in vivo*

Only few studies so far have investigated the anti-proliferative and tumor growth inhibiting potential of XN in animal models. In the course of a study by our group [9,21], intravital microscopy was used to investigate the effect of XN on tumor angiogenesis and tumor growth *in vivo*. Human MX-1 breast tumor xenografts were implanted in dorsal skinfold chamber preparations in female Severe Combined Immuno-Deficient (SCID) mice. Starting from day 15 after tumor implantation, animals were treated with XN applied subcutaneously at a dose of 1000 mg/kg body weight per day or DMSO as solvent control, respectively, for 7 and 14 days. To assess tumor growth, the two-dimensional tumor surface was documented with digital photography using bright field microscopy. Functional vessel density was quantified by intravital fluorescence video microscopy after injection of FITC-labelled dextran. Application of XN for 7 and 14 days inhibited the growth of the breast tumor xenografts by 46% and 83%, respectively, in comparison with the solvent-treated control group, and reduced the size of established tumors by 30 and 56%, respectively. Concomitantly, XN-treatment for 14 days reduced tumor-induced neovascularization by 33% [9,21].

Albini *et al.* tested the effect of XN applied orally at a dose of 20 μ M in drinking water on the growth of Karposi's sarcoma xenografts [15]. KS-IMM cells mixed with matrigel were injected on the flanks of 7 week old *nu/nu* (CD-1)BR mice. XN application was started 4 days before cell injection. 24 days after injection, the average tumor volume was significantly reduced by 70% by XN intervention. The average tumor weight was also significantly inhibited by about 45%. Inhibition of

tumor growth was accompanied by a reduction of vascularisation and extensive areas of necrosis and fibrosis in comparison with control tumors [15].

In a third investigation, Monteiro *et al.* tested the effect of orally applied XN on the growth of MCF-7 breast cancer xenografts in male nude mice. XN was applied at a dose of 100 μ M in 0.1% ethanol as drinking source for 60 days. In comparison with the solvent control, XN treatment non-significantly reduced average tumor weights. Morphologically, tumors from XN-treated animals showed large areas of necrosis, a decrease in the number of infiltrating inflammatory cells, and decreased NF- κ B, phosphorylated I κ B, and cytokine IL1 β staining. XN treatment also reduced cell proliferation assessed by Ki67 staining from a diffuse pattern in control tumors to focal areas of proliferating cells, and doubled the number of TUNEL-positive cells as an indication of apoptosis induction. Consistent with previous studies, tumors of XN-treated animals presented significantly lower microvessel density than tumors of control mice. Also, expression of factor VIII as an endothelial marker was significantly reduced.

6 Summary and Conclusion

As outlined above, XN is a natural product with a broad spectrum of biological activities. There is consistent evidence from *in vitro* studies that XN inhibits cell proliferation by inhibition of DNA synthesis, induction of cell cycle arrest and induction of apoptosis. Apoptosis induction is observed in a wide panel of cell types and in cancer cells derived from a large spectrum of tumor sites. Primary cells appear to be less sensitive to the anti-proliferative activity of XN than transformed cells.

XN induces apoptosis by activation of the death receptor-mediated extrinsic as well as the mitochondria-mediated intrinsic pathway. Inhibition of TNF-mediated activation of NF- κ B by XN has been associated with induction of apoptosis *via* the extrinsic pathway in various studies. In cell culture, XN treatment results in an immediate transient increase in O₂^{•-} generation by inhibition of the mitochondrial respiratory chain. This increase in oxidative stress is considered as the trigger of apoptosis induction. Recent evidence indicates that XN induces ER stress as an additional mechanism of apoptosis induction, which might also be activated by ROS production.

Only few studies so far have addressed the question of whether XN reduces tumor growth *in vivo*. Inhibition of mammary cancer and Kaposi's sarcoma xenograft growth by XN has consistently been related to the inhibition of angiogenesis. There is limited evidence that induction of apoptosis also contributes to tumor growth inhibition. The mechanisms of apoptosis induction *in vivo* may involve prevention of TNF-induced NF- κ B activity.

7 References

1. Hanahan D, Weinberg RA (2000) The hallmarks of cancer. *Cell* 100 (1):57-70
2. Stevens JF, Ivancic M, Hsu VL, Deinzer ML (1997) Prenylflavonoids from *Humulus lupulus*. *Phytochemistry* 44 (8):1575-1585
3. Henderson MC, Miranda CL, Stevens JF, Deinzer ML, Buhler DR (2000) In vitro inhibition of human P450 enzymes by prenylated flavonoids from hops, *Humulus lupulus*. *Xenobiotica* 30 (3):235-251
4. Miranda CL, Aponso GL, Stevens JF, Deinzer ML, Buhler DR (2000) Prenylated chalcones and flavanones as inducers of quinone reductase in mouse Hepa 1c1c7 cells. *Cancer Lett* 149 (1-2):21-29
5. Miranda CL, Stevens JF, Ivanov V, McCall M, Frei B, Deinzer ML, Buhler DR (2000) Antioxidant and prooxidant actions of prenylated and nonprenylated chalcones and flavanones in vitro. *Journal of Agricultural and Food Chemistry* 48 (9):3876-3884. doi:Doi 10.1021/Jf0002995
6. Miranda CL, Stevens JF, Helmrich A, Henderson MC, Rodriguez RJ, Yang YH, Deinzer ML, Barnes DW, Buhler DR (1999) Antiproliferative and cytotoxic effects of prenylated flavonoids from hops (*Humulus lupulus*) in human cancer cell lines. *Food Chem Toxicol* 37 (4):271-285. doi:S0278691599000198 [pii]
7. Gerhauser C, Alt A, Heiss E, Gamal-Eldeen A, Klimo K, Knauff J, Neumann I, Scherf HR, Frank N, Bartsch H, Becker H (2002) Cancer chemopreventive activity of Xanthohumol, a natural product derived from hop. *MolCancer Ther* 1 (11):959-969
8. Stevens JF, Page JE (2004) Xanthohumol and related prenylflavonoids from hops and beer: to your good health! *Phytochemistry* 65 (10):1317-1330
9. Gerhauser C (2005) Beer constituents as potential cancer chemopreventive agents. *EurJCancer* 41 (13):1941-1954
10. Zanoli P, Zavatti M (2008) Pharmacognostic and pharmacological profile of *Humulus lupulus* L. *JEthnopharmacol* 116 (3):383-396
11. Magalhaes PJ, Carvalho DO, Cruz JM, Guido LF, Barros AA (2009) Fundamentals and health benefits of xanthohumol, a natural product derived from hops and beer. *NatProdCommun* 4 (5):591-610
12. Chadwick LR, Pauli GF, Farnsworth NR (2006) The pharmacognosy of *Humulus lupulus* L. (hops) with an emphasis on estrogenic properties. *Phytomedicine* 13 (1-2):119-131
13. Strathmann J, Klimo K, Steinle R, Hussong R, Frank N, Gerhauser C (2009) Xanthohumol from Hops Prevents Hormone-dependent Tumorigenesis in vitro and in vivo. *Acta Horticulturae Proceedings of the Second International Humulus Symposium* (848):179-190
14. Botta B, Vitali A, Menendez P, Misiti D, Delle MG (2005) Prenylated flavonoids: pharmacology and biotechnology. *CurrMedChem* 12 (6):717-739
15. Albin A, Dell'Eva R, Vene R, Ferrari N, Buhler DR, Noonan DM, Fassina G (2006) Mechanisms of the antiangiogenic activity by the hop flavonoid xanthohumol: NF-kappaB and Akt as targets. *FASEB J* 20 (3):527-529

16. Colgate EC, Miranda CL, Stevens JF, Bray TM, Ho E (2007) Xanthohumol, a prenylflavonoid derived from hops induces apoptosis and inhibits NF-kappaB activation in prostate epithelial cells. *Cancer Lett* 246 (1-2):201-209
17. Dell'Eva R, Ambrosini C, Vannini N, Piaggio G, Albini A, Ferrari N (2007) AKT/NF-kappaB inhibitor xanthohumol targets cell growth and angiogenesis in hematologic malignancies. *Cancer* 110 (9):2007-2011
18. Harikumar KB, Kunnumakkara AB, Ahn KS, Anand P, Krishnan S, Guha S, Aggarwal BB (2009) Modification of the cysteine residues in IkappaBalpha kinase and NF-kappaB (p65) by xanthohumol leads to suppression of NF-kappaB-regulated gene products and potentiation of apoptosis in leukemia cells. *Blood* 113 (9):2003-2013
19. Guerreiro S, Monteiro R, Martins MJ, Calhau C, Azevedo I, Soares R (2007) Distinct modulation of alkaline phosphatase isoenzymes by 17beta-estradiol and xanthohumol in breast cancer MCF-7 cells. *ClinBiochem* 40 (3-4):268-273
20. Monteiro R, Becker H, Azevedo I, Calhau C (2006) Effect of hop (*Humulus lupulus* L.) flavonoids on aromatase (estrogen synthase) activity. *J Agric Food Chem* 54 (8):2938-2943
21. Klenke E (2008) *Inhibition of Angiogenesis by Potential Chemopreventive Agents*. vol 1. Vdm Verlag Dr. Müller,
22. Goto K, Asai T, Hara S, Namatame I, Tomoda H, Ikemoto M, Oku N (2005) Enhanced antitumor activity of xanthohumol, a diacylglycerol acyltransferase inhibitor, under hypoxia. *Cancer Lett* 219 (2):215-222. doi:S0304-3835(04)00577-4 [pii] 10.1016/j.canlet.2004.07.034
23. Monteiro R, Faria A, Azevedo I, Calhau C (2007) Modulation of breast cancer cell survival by aromatase inhibiting hop (*Humulus lupulus* L.) flavonoids. *J Steroid Biochem Mol Biol* 105 (1-5):124-130. doi:S0960-0760(07)00105-7 [pii] 10.1016/j.jsbmb.2006.11.026
24. Ho YC, Liu CH, Chen CN, Duan KJ, Lin MT (2008) Inhibitory effects of xanthohumol from hops (*Humulus lupulus* L.) on human hepatocellular carcinoma cell lines. *Phytotherapy Research* 22 (11):1465-1468. doi:10.1002/ptr.2481
25. Dorn C, Kraus B, Motyl M, Weiss TS, Gehrig M, Scholmerich J, Heilmann J, Hellerbrand C (2010) Xanthohumol, a chalcon derived from hops, inhibits hepatic inflammation and fibrosis. *Mol Nutr Food Res* 54 Suppl 2:S205-213. doi:10.1002/mnfr.200900314
26. Dorn C, Weiss TS, Heilmann J, Hellerbrand C (2010) Xanthohumol, a prenylated chalcone derived from hops, inhibits proliferation, migration and interleukin-8 expression of hepatocellular carcinoma cells. *Int J Oncol* 36 (2):435-441
27. Jin Z, El-Deiry WS (2005) Overview of cell death signaling pathways. *Cancer Biol Ther* 4 (2):139-163. doi:1508 [pii]
28. Faitova J, Krekac D, Hrstka R, Vojtesek B (2006) Endoplasmic reticulum stress and apoptosis. *Cell Mol Biol Lett* 11 (4):488-505. doi:10.2478/s11658-006-0040-4
29. Heath-Engel HM, Chang NC, Shore GC (2008) The endoplasmic reticulum in apoptosis and autophagy: role of the BCL-2 protein family. *Oncogene* 27 (50):6419-6433. doi:onc2008309 [pii]

10.1038/onc.2008.309

30. Carpinteiro A, Dumitru C, Schenck M, Gulbins E (2008) Ceramide-induced cell death in malignant cells. *Cancer Lett* 264 (1):1-10. doi:S0304-3835(08)00082-7 [pii] 10.1016/j.canlet.2008.02.020

31. Grassme H, Cremesti A, Kolesnick R, Gulbins E (2003) Ceramide-mediated clustering is required for CD95-DISC formation. *Oncogene* 22 (35):5457-5470. doi:10.1038/sj.onc.1206540
1206540 [pii]

32. Ak P, Levine AJ (2010) p53 and NF-kappaB: different strategies for responding to stress lead to a functional antagonism. *FASEB J* 24 (10):3643-3652. doi:fj.10-160549 [pii] 10.1096/fj.10-160549

33. Pan L, Becker H, Gerhauser C (2005) Xanthohumol induces apoptosis in cultured 40-16 human colon cancer cells by activation of the death receptor- and mitochondrial pathway. *MolNutrFood Res* 49 (9):837-843

34. Vanhoecke B, Derycke L, Van Marck V, Depypere H, De Keukeleire D, Bracke M (2005) Antiinvasive effect of xanthohumol, a prenylated chalcone present in hops (*Humulus lupulus* L.) and beer. *International Journal of Cancer* 117 (6):889-895. doi:10.1002/ijc.21249

35. Lust S, Vanhoecke B, Janssens A, Philippe J, Bracke M, Offner F (2005) Xanthohumol kills B-chronic lymphocytic leukemia cells by an apoptotic mechanism. *Molecular Nutrition & Food Research* 49 (9):844-850. doi:10.1002/mnfr.200500045

36. Xuan NT, Shumilina E, Gulbins E, Gu S, Götz F, Lang F (2010) Triggering of dendritic cell apoptosis by xanthohumol. *Molecular Nutrition & Food Research* 54 (S2):S214-S224. doi:10.1002/mnfr.200900324

37. Monteghirfo S, Tosetti F, Ambrosini C, Stigliani S, Pozzi S, Frassoni F, Fassina G, Soverini S, Albini A, Ferrari N (2008) Antileukemia effects of xanthohumol in Bcr/Abl-transformed cells involve nuclear factor-kappaB and p53 modulation. *MolCancer Ther* 7 (9):2692-2702

38. Szliszka E, Czuba ZP, Mazur B, Paradysz A, Krol W (2010) Chalcones and dihydrochalcones augment TRAIL-mediated apoptosis in prostate cancer cells. *Molecules* 15 (8):5336-5353. doi:molecules15085336 [pii] 10.3390/molecules15085336

39. Lust S, Vanhoecke B, Van GM, Boelens J, Van MH, Kaileh M, Vanden Berghe W, Haegeman G, Philippe J, Bracke M, Offner F (2009) Xanthohumol activates the proapoptotic arm of the unfolded protein response in chronic lymphocytic leukemia. *Anticancer Res* 29 (10):3797-3805

40. Kamata H, Hirata H (1999) Redox regulation of cellular signalling. *Cell Signal* 11 (1):1-14

41. Boveris A, Chance B (1973) The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *BiochemJ* 134 (3):707-716

42. Boveris A, Cadenas E (1975) Mitochondrial production of superoxide anions and its relationship to the antimycin insensitive respiration. *FEBS Lett* 54 (3):311-314

43. Fruehauf JP, Meyskens FL, Jr. (2007) Reactive oxygen species: a breath of life or death? *ClinCancer Res* 13 (3):789-794
44. Pani G, Koch OR, Galeotti T (2009) The p53-p66shc-Manganese Superoxide Dismutase (MnSOD) network: A mitochondrial intrigue to generate reactive oxygen species. *The International Journal of Biochemistry & Cell Biology* 41 (5):1002-1005
45. Green DR, Reed JC (1998) Mitochondria and apoptosis. *Science* 281 (5381):1309-1312
46. Desagher S, Martinou JC (2000) Mitochondria as the central control point of apoptosis. *Trends Cell Biol* 10 (9):369-377
47. Orrenius S, Gogvadze V, Zhivotovsky B (2007) Mitochondrial oxidative stress: implications for cell death. *AnnuRevPharmacolToxicol* 47:143-183
48. Simon HU, Haj-Yehia A, Levi-Schaffer F (2000) Role of reactive oxygen species (ROS) in apoptosis induction. *Apoptosis* 5 (5):415-418
49. Trachootham D, Alexandre J, Huang P (2009) Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? *NatRevDrug Discov* 8 (7):579-591
50. Yang JY, la-Fera MA, Rayalam S, Baile CA (2007) Effect of xanthohumol and isoxanthohumol on 3T3-L1 cell apoptosis and adipogenesis. *Apoptosis* 12 (11):1953-1963
51. Strathmann J, Klimo K, Sauer SW, Okun JG, Prehn JHM, Gerhauser C (2010) Xanthohumol-induced transient superoxide anion radical formation triggers cancer cells into apoptosis via a mitochondria-mediated mechanism. *FASEB J* 24 (8):2938-2950. doi:10.1096/fj.10-155846
52. Halliwell B, Whiteman M (2004) Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? *BrJPharmacol* 142 (2):231-255
53. Zhao H, Joseph J, Fales HM, Sokoloski EA, Levine RL, Vasquez-Vivar J, Kalyanaraman B (2005) Detection and characterization of the product of hydroethidine and intracellular superoxide by HPLC and limitations of fluorescence. *Proceedings of the National Academy of Sciences of the United States of America* 102 (16):5727-5732
54. Malhotra JD, Kaufman RJ (2007) Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? *Antioxid Redox Signal* 9 (12):2277-2293. doi:10.1089/ars.2007.1782
55. Zhang K, Kaufman RJ (2008) From endoplasmic-reticulum stress to the inflammatory response. *Nature* 454 (7203):455-462. doi:nature07203 [pii] 10.1038/nature07203
56. Lee SH, Kim HJ, Lee JS, Lee IS, Kang BY (2007) Inhibition of topoisomerase I activity and efflux drug transporters' expression by xanthohumol. *Arch Pharm Res* 30 (11):1435-1439
57. Monteiro R, Calhau C, Silva AO, Pinheiro-Silva S, Guerreiro S, Gartner F, Azevedo I, Soares R (2008) Xanthohumol inhibits inflammatory factor production and angiogenesis in breast cancer xenografts. *JCell Biochem* 104 (5):1699-1707

58. Vogel S, Heilmann J (2008) Synthesis, cytotoxicity, and antioxidative activity of minor prenylated chalcones from *Humulus lupulus*. *J Nat Prod* 71 (7):1237-1241. doi:10.1021/np800188b
59. Vogel S, Ohmayer S, Brunner G, Heilmann J (2008) Natural and non-natural prenylated chalcones: synthesis, cytotoxicity and anti-oxidative activity. *Bioorg Med Chem* 16 (8):4286-4293. doi:S0968-0896(08)00198-3 [pii]
10.1016/j.bmc.2008.02.079
60. Vogel S, Barbic M, Jurgenliemk G, Heilmann J (2010) Synthesis, cytotoxicity, anti-oxidative and anti-inflammatory activity of chalcones and influence of A-ring modifications on the pharmacological effect. *Eur J Med Chem* 45 (6):2206-2213. doi:S0223-5234(10)00105-4 [pii]
10.1016/j.ejmech.2010.01.060
61. Delmulle L, Vanden Berghe T, Keukeleire DD, Vandenabeele P (2008) Treatment of PC-3 and DU145 prostate cancer cells by prenylflavonoids from hop (*Humulus lupulus* L.) induces a caspase-independent form of cell death. *Phytother Res* 22 (2):197-203. doi:10.1002/ptr.2286
62. Yang JY, Della-Fera MA, Rayalam S, Baile CA (2008) Enhanced effects of xanthohumol plus honokiol on apoptosis in 3T3-L1 adipocytes. *Obesity (Silver Spring)* 16 (6):1232-1238. doi:oby200866 [pii]
10.1038/oby.2008.66
63. Rayalam S, Yang JY, Della-Fera MA, Park HJ, Ambati S, Baile CA (2009) Anti-obesity effects of xanthohumol plus guggulsterone in 3T3-L1 adipocytes. *J Med Food* 12 (4):846-853. doi:10.1089/jmf.2008.0158
64. Mendes V, Monteiro R, Pestana D, Teixeira D, Calhau C, Azevedo I (2008) Xanthohumol influences preadipocyte differentiation: implication of antiproliferative and apoptotic effects. *J Agric Food Chem* 56 (24):11631-11637. doi:10.1021/jf802233q
10.1021/jf802233q [pii]
65. Gao X, Deeb D, Liu Y, Gautam S, Dulchavsky SA, Gautam SC (2009) Immunomodulatory activity of xanthohumol: inhibition of T cell proliferation, cell-mediated cytotoxicity and Th1 cytokine production through suppression of NF-kappaB. *Immunopharmacol Immunotoxicol* 31 (3):477-484. doi:10.1080/08923970902798132

Annex (Table 1)**Table 1.** Anti-proliferative and apoptosis-inducing potential of XN *in vitro*

Organ	Cell lines	Xanthohumol concentration	Treatment time	Effect	Ref.
Ovary	A-2780	0.1-100 μ M	48, 96h	\downarrow proliferation	[6]
Ovary	SK-OV-3	not stated	48h	\downarrow proliferation	[56]
Breast	MCF7	0. 1-100 μ M	48, 96h	\downarrow proliferation	[6]
		0.01-100 μ M	8, 24, 48h	cytotoxicity at 100 μ M	
		0.1-100 μ M	24-96h	\downarrow DNA synthesis	
		10, 40, 100 μ M	8, 24h	\leftrightarrow induction of apoptosis	
Breast	MDA-MB-435	3-50 μ M	72h	\downarrow proliferation	[7]
		3-50 μ M	72h	\downarrow DNA synthesis	
		5-50 μ M	24h	S-phase cell cycle arrest	
Breast	MCF-7; T47D	1-20 μ M	8 days	\downarrow proliferation	[34]
	MCF-7	10 μ M	96h	\uparrow nuclear condensation	
	T47D	5-25 μ M	48h	\uparrow PARP cleavage	
Breast	Sk-Br-3	0.005-25 μ M	72h	\downarrow proliferation	[23]
		0.005-25 μ M	72h	\downarrow DNA synthesis	
		5 μ M	24h	\downarrow proliferation	
		5 μ M	24h	\uparrow apoptosis	
Breast	MCF-7	10 μ M	24h	\downarrow viable cells	[19]
		10 μ M	24h	\downarrow proliferation	
		10 μ M	24h	\downarrow apoptosis	
Breast	MCF7	0.1-100 μ M	24, 72h	\downarrow viable cells; \uparrow cytotoxicity	[57]
		0.1-100 μ M	24, 72h	\downarrow DNA synthesis	
		10 μ M	24h	\downarrow proliferation	
Endo-metrium	Ishikawa	1.6-50 μ M	72h	\downarrow proliferation	[7]
Cervix	HeLa	not stated	72h	\downarrow proliferation	[58]
					[59]
					[60]
					[16]
Prostate	BPH-1*/PC-3	2.5-20 μ M	48h	\downarrow proliferation	
		10, 20 μ M	48h	\uparrow cell death	
		10, 20 μ M	48h	\uparrow caspase activity	
	BPH-1	10, 20 μ M	48h	S-phase cell cycle arrest	
		10, 20 μ M	48h	\downarrow NF- κ B activity	
			\uparrow p65 nuclear translocation		

		10, 20 μ M	48h	\uparrow expression of Bax, p53 \downarrow Bcl-2 expression	
Prostate	PC-3, DU145	100, 200 μ M 200 μ M	2h up to 2h	\downarrow cell viability \leftrightarrow caspase 3 activation	[61]
Prostate	LNCaP	20-50 μ M + TRAIL	48h	\uparrow cytotoxicity in combination with TRAIL	[38]
		20, 50 μ M + TRAIL		synergistic \uparrow apoptosis no necrotic cell death	
Prostate	BPH-1	1.6-50 μ M	72h	\downarrow proliferation	[51]
		1.6-50 μ M	5-45min	\uparrow ROS production	
		3.1-50 μ M	10-30min	\downarrow Ψ_m	
		6.3-50 μ M	30min- 24h	\uparrow cytochrome <i>c</i> release	
		10-40 μ M	48h	\uparrow PARP cleavage	
Colon	HT-29	10-40 μ M	72h	\uparrow apoptosis (sub-G ₁ fraction)	
Colon	HCT116 cl. 40-16	0.01-100 μ M	48, 96h	cytotoxicity at 100 μ M	[6]
		0.5-10 μ M	72h	\downarrow proliferation \uparrow PARP cleav- age	[33]
		5-15 μ M	24-72h	\uparrow caspase-3, -7, -8, -9 cleav- age	
		5-15 μ M	24-72h	\downarrow Bcl-2 expression	
Colon	HCT15	not stated	48h	\downarrow proliferation	[56]
Liver	primary rat hepatocytes	0.1-100 μ M	24h	cytotoxicity at 100 μ M	[6]
Liver	Hepa1c1c7	0.4-25 μ M	48h	\downarrow proliferation	[7]
Liver	AML12*	10-225 μ M	24h	\downarrow proliferation	[24]
	HA22T/VGH HEP3B				
	HA22T/VGH HEP3B	90, 135 μ M 90 μ M	4h 24h	\uparrow apoptosis \uparrow nuclear condensation	
		45, 90, 135 μ M	24h	\uparrow DNA fragmentation	
Liver	HSC ^a	0-40 μ M	6h	\uparrow caspase-3 activity	[25]
		5, 10, 20 μ M	24h	\uparrow apoptosis/necrosis	
		5-40 μ M	24h	\downarrow vitality	
		5 μ M	2h	\downarrow basal and TNF-induced NF- κ B activity	
	PHH*	25, 50 μ M	24h	\leftrightarrow vitality	
		25, 50 μ M	24h	\leftrightarrow apoptosis/necrosis	
		25, 50 μ M	24h	\downarrow basal and palmitate-induced pro-inflammatory IL-8 mRNA expression	
Liver	HepG2, Huh7	10-100 μ M	24h	\downarrow proliferation \uparrow caspase-3 ac-	[26]

		25 μ M		tivity	
	Huh7	2.5 μ M	3h preinc.	\downarrow TNF-induced NF- κ B activity and IL-8 mRNA expression	
Lung	PHH	10-100 μ M	24h	no inhibition of cell viability	
	A549	not stated	48h	\downarrow proliferation	[56]
Leukemia	HL-60	0.5-10 μ M	72h	\downarrow proliferation	[7]
Leukemia	primary B-CLL cells	10, 25 μ M	24, 48h	\uparrow cell death/apoptosis	[35]
		25 μ M	24, 48h	\uparrow PARP cleavage	
Leukemia	MM6, U937	2.5-10 μ M	24 -72h	\downarrow proliferation	[17]
	primary AML and CLL cells	2.5-10 μ M	24 -72h	\uparrow cell death	
		5 μ M	6h	\downarrow TNF-induced NF- κ B signaling	
Leukemia	K562	2.5-10 μ M	24-72h	\downarrow proliferation	[37]
	primary CML cells	5 μ M	24h	\uparrow apoptosis	
		5 μ M	6h	\uparrow ROS, \downarrow TNF-induced NF- κ B signalling, \uparrow p21, p53 mRNA and protein expression	
		5 μ M	16, 24h	\downarrow survivin mRNA and protein expression	
Leukemia	primary CLL cells	25 μ M	12, 24h	\uparrow caspase-3, -4, -9 cleavage	[39]
		25 μ M	3-24h	\downarrow Bid, Mcl-1 expression	
		25 μ M	24h	\downarrow Bcl-2 expression	
		25 μ M	12, 24h	\uparrow ER-stress, \uparrow UPR	
		25 μ M	12h	\uparrow ROS	
		25-100 μ M		\downarrow S20 proteasomal activity	
		25 μ M	12h	\uparrow ubiquitinated proteins	
Leukemia	KBM-5 (CML)	50 μ M	4h	\uparrow TNF-induced apoptosis	[18]
		50 μ M	12, 24h	\uparrow TNF-induced PARP cleavage	
		50 μ M	4h+0-12h	\downarrow expression of TNF-induced proliferative and anti-apoptotic proteins	
		50 μ M/ 5-50 μ M	1-12h/4h	\downarrow TNF-induced activation of NF- κ B, I κ B α kinase activity	
Myeloma	U266	50 μ M	4h	\uparrow TNF-induced apoptosis	[18]
Sarcoma	HT-1080	3 μ M	72h	\downarrow proliferation under hypoxia	[22]
Sarcoma	KS-IMM	2.5-25 μ M	48-72h	\downarrow proliferation	[15]
Melanoma	SK-Mel2	not stated	48h	\downarrow proliferation	[56]
Macro-	Raw264.7	0.4-50 μ M	24h	\leftrightarrow proliferation	[7]

phages					
Endothelial cells	HUVEC	2.5-25 μ M	48-72h	↓ proliferation (10 – 15 μ M) ↑ cell death (25 μ M)	[15]
		10 μ M	3h pre-treatment	↓ TNF-induced NF- κ B nuclear translocation, ↓ I κ B α phosphorylation	
Endothelial cells	HUVEC	2.5-10 μ M	24-72h	↓ proliferation	[17]
Adipocytes	from 3T3-L1	25-100 μ M	24, 48h	↓ proliferation	[50] [62] [63]
		25-100 μ M	24, 48h	↑ apoptosis	
		75 μ M	0-3h	↑ ROS production	
		75 μ M	0-1.5h	↓ mitochondrial membrane potential	
		75 μ M	6-48h	↑ cytochrome <i>c</i> release	
		75, 100 μ M	3-12h	↑ caspase3/7 activity	
		75 μ M	6-24h	↑ PARP cleavage	
Adipocytes	from 3T3-L1	0.1-50 μ M	24-72h	↓ proliferation	[64]
		5 μ M	24h	↓ proliferation	
		5 μ M	24h	↑ apoptosis	
Dendritic cells	from bone marrow	2-50 μ M	24h	↑ caspase-3, -8 activity	[36]
		20 μ M	24h	↑ caspase-3, -8 cleavage	
		2-50 μ M	24h	↑ apoptosis (sub-G ₁ fraction)	
T-cells	murine T-lymphocytes	1.25-40 μ M	72, 96h	↓ Con A or IL-2-induced proliferation	[65]
		1.25-40 μ M	72h	↓ cell viability at high, ↑ cell counts at low concentrations	

^aHSC, hepatic stellate cells

*non-cancerous cell lines: AML12, normal murine hepatocyte cell line; PHH, primary human hepatocytes; BPH-1, benign prostatic hyperplasia (prostate epithelial cells)