Chapter 4
Anti-proliferative and Apoptosis-Inducing Properties of Xanthohumol, a Prenylated Chalcone from Hops (*Humulus lupulus* L.)

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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-κB 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

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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
H_2O_2	hydrogen peroxide
HUVEC	human umbilical vein endothelial cells
IC_{50}	half-maximal inhibitory concentration
IKK	I-κB kinase
IL	interleukin
Ire1α	inositol-requiring 1α
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-κB	nuclear factor κB
$O_2^{-\bullet}$	superoxide anion radicals
$OH^{\bullet}$	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
XBP1	X-box-binding protein 1
XN	Xanthohumol
Ψ_m	mitochondrial membrane potential
ρ^0	rho zero

4.1 Introduction

Evading apoptosis has been recognized as one of the hallmarks of cancer cells (Hanahan and Weinberg 2000). 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. 4.1), a prenylated chalcone found in hops (*Humulus lupulus* L.).

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) (Stevens et al. 1997).

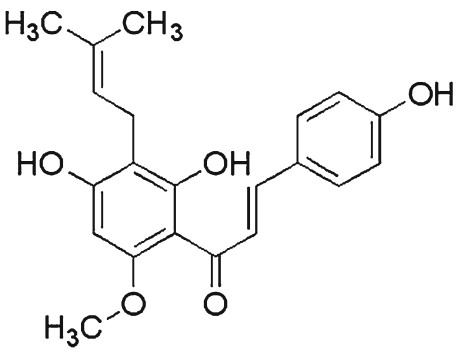

Fig. 4.1 Chemical structure of xanthohumol (2′,4,4′-trihydroxy-3′-prenyl-6′-methoxychalcone)

4.2 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* (Henderson et al. 2000; Miranda et al. 2000a) and to possess antioxidant (Miranda et al. 2000b), anti-proliferative and cytotoxic activity (Miranda et al. 1999). Subsequently, we identified XN as a broad spectrum cancer chemopreventive agent able to interfere with the initiation, promotion and progression phase of carcinogenesis (Gerhauser et al. 2002). Several recent reviews have comprehensively summarized the cancer preventive and health promoting activities of XN and other hops components (Gerhauser et al. 2002; Stevens and Page 2004; Gerhauser 2005; Zanoli and Zavatti 2008; Magalhaes et al. 2009; Chadwick et al. 2006; Strathmann et al. 2009; Botta et al. 2005). 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 (Henderson et al. 2000; Gerhauser et al. 2002). Antioxidant and radical scavenging properties can also contribute to the inhibition of tumor initiation by XN (Gerhauser et al. 2002; reviewed in Gerhauser 2005; Strathmann et al. 2009). XN inhibits cyclooxygenases (Cox)-1 and -2 activities as well as the production of nitric oxide (Gerhauser et al. 2002). 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 κB (NF-κB) signaling and subsequent downregulation of pro-inflammatory key factors (Albini et al. 2006; Colgate et al. 2007; Dell'Eva et al. 2007; Harikumar et al. 2009). 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 (Gerhauser et al. 2002; Guerreiro et al. 2007). XN also inhibits the enzyme aromatase (CYP19), which plays a crucial role in the conversion of testosterone to estrogen (Strathmann et al. 2009; Monteiro et al. 2006). In the progression phase, XN affects cell proliferation by induction of cell differentiation (Gerhauser et al. 2002) 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 endothelia cell migration and vessel formation using human microendothelial cells (Gerhauser 2005). *In vivo*, inhibition of angiogenesis was demonstrated in human breast cancer xenografts in a

skinfold chamber model (Klenke 2008), and with a matrigel sponge angiogenesis assay as described by Albini et al. (2006). 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 (Gerhauser et al. 2002). 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).

4.3 Cell-Growth Inhibitory Potential of Xanthohumol

The first indication of anti-proliferative potential of XN was reported by Miranda et al. (1999). 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 (IC_{50} values) of cell viability in the range of 0.5–25 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 (Goto et al. 2005). 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 (Monteiro et al. 2007). Importantly, primary hepatocytes were more resistant to the anti-proliferative effects of XN than liver cancer cells (Ho et al. 2008; Dorn et al. 2010a, b).

4.4 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 (Jin and El-Deiry 2005). In addition, apoptosis can be triggered by endoplasmatic reticulum (ER) stress and unfolded protein response (Faitova et al. 2006; Heath-Engel et al. 2008). There is accumulating evidence that XN targets all three pathways.

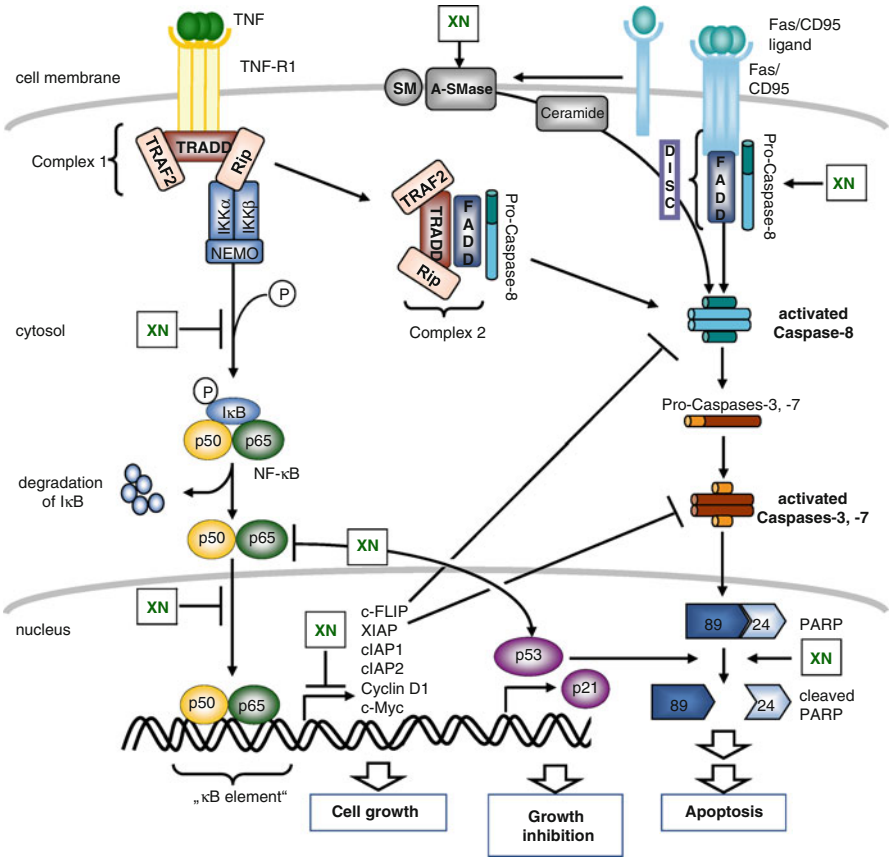

Fig. 4.2 Induction of apoptosis via the death receptor pathway, and the influence of XN on these mechanisms (see text for description)

4.4.1 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. 4.2, 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 Jin and El-Deiry 2005). 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 (Carpinteiro et al. 2008). 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 (Grassme et al. 2003).

TNF is a multifunctional pro-inflammatory protein that activates the NF-κB pathway through binding to TNF-receptors (Fig. 4.2, 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 phosphorylation and subsequent ubiquitin-mediated degradation of its inhibitor I-κB by I-κB kinase (IKK), which is recruited to the TNF-R1 complex through TRAF2 and stabilized by RIP. Degradation of I-κB liberates NF-κB and allows nuclear translocation, where it interacts with the κB 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-κB mediates a strong pro-survival mechanism. NF-κB is involved in the regulation of gene expression of about 200 genes involved in inflammation, development, cell growth, and inhibition of apoptosis (Ak and Levine 2010). Consequently, the cell-death inducing capacity of TNF is only apparent when NF-κB activation is blocked. Ak and Levine have recently postulated that NF-κB and p53 have mutually exclusive functions: inactivation of NF-κB enhances the stability of p53, which then contributes to cell growth inhibition and apoptosis induction (Ak and Levine 2010).

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 (Pan et al. 2005). 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 (Vanhoecke et al. 2005; Lust et al. 2005) (Table 1 in the Annex). Interestingly, Xuan et al. recently identified a role of A-SMase-derived ceramide in XN-mediated apoptosis induction in dendritic cells (DC) (Xuan et al. 2010).

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-κB 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-κB, which was completely blocked by pretreatment with 10 μM XN. This was attributed to the inhibition I-κB phosphorylation by XN. In addition to blocking TNF-induced NF-κB activation, XN inhibited the constitutive activity of NF-κB 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-κB nuclear translocation. Still, caspases were activated, and XN treatment resulted in cell growth inhibition and cell death (Colgate et al. 2007). In MM6 and U937 leukemia cells stimulated with TNF, inhibition of NF-κB 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 (Dell'Eva et al. 2007). 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-κB. XN treatment reduced cell viability and led to apoptosis induction. Also, cell invasion was reduced. Both effects were attributed to suppression of NF-κB activation as well as IκB 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 (Monteghirfo et al. 2008). Most of these XN-mediated effects on cell proliferation, apoptosis induction, Bcr-Abl expression, and NF-κB 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-κB signaling. XN treatment blocked constitutive NF-κB activity and NF-κB activation by TNF, prevented nuclear translocation of the NF-κB p65 subunit to the nucleus, suppressed NF-κB-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 IκB kinase (IKK) and the p65 subunit of NF-κB through its unsaturated ketone moiety (Harikumar et al. 2009). Inhibition of NF-κB activation by XN and increased caspases 3 activity was also involved in apoptosis induction in hepatic stellate cells and Huh7 human liver cancer cells (Dorn et al. 2010a, b). 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 (Szliszka et al. 2010).

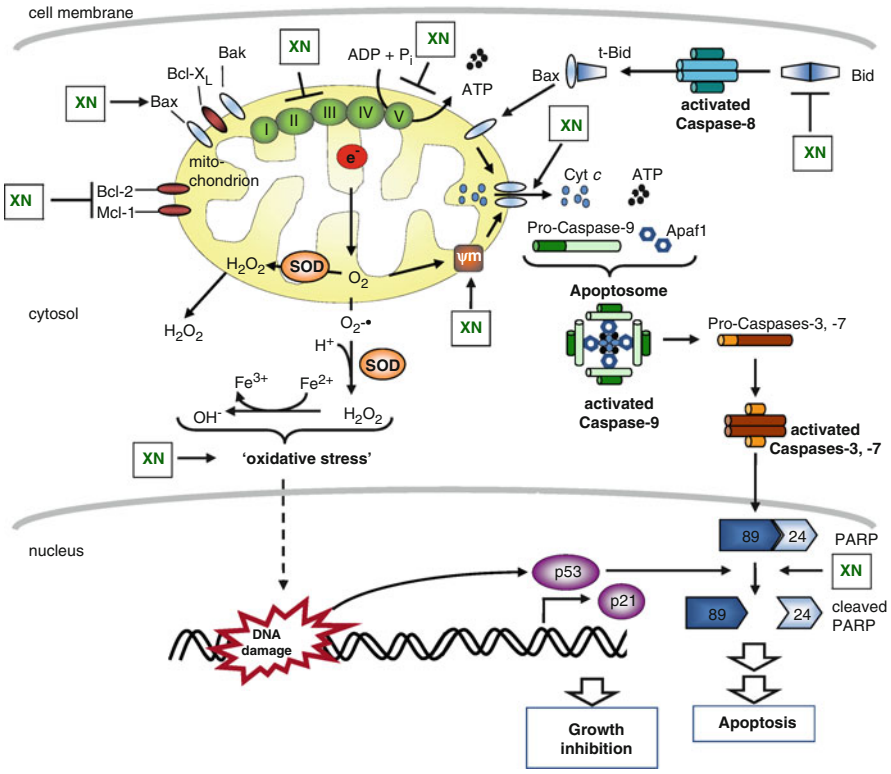

Fig. 4.3 Induction of apoptosis via the mitochondrial pathway, including the role of reactive oxygen species (*ROS*). Mechanisms targeted by XN are indicated (see text for description)

4.4.2 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. 4.3). 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 (Jin and El-Deiry 2005).

Our study with 40–16 colon cancer cells indicated that XN not only activates the death receptor pathway, but also the intrinsic apoptosis pathway (Pan et al. 2005). 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 (Colgate et al. 2007). 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 (Lust et al. 2009).

4.4.3 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 (H_2O_2), superoxide anion radicals ($O_2^{-\bullet}$) and hydroxyl radicals ($OH^{\bullet}$) being the most abundant ones (Kamata and Hirata 1999). 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 $O_2^{-\bullet}$ (Boveris and Chance 1973; Boveris and Cadenas 1975; Fruehauf and Meyskens 2007; Pani et al. 2009).

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 (Green and Reed 1998; Desagher and Martinou 2000). Impaired mitochondrial functions suppress mitochondrial metabolism, imbalance the mitochondrial membrane potential, block respiration and oxidative phosphorylation (Orrenius et al. 2007), finally leading to apoptosis induction (Simon et al. 2000). 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 (Trachootham et al. 2009).

Despite its antioxidant activity (Gerhauser et al. 2002), several recent reports suggest that induction of apoptosis by XN is linked to the induction of ROS (Fig. 4.3). 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

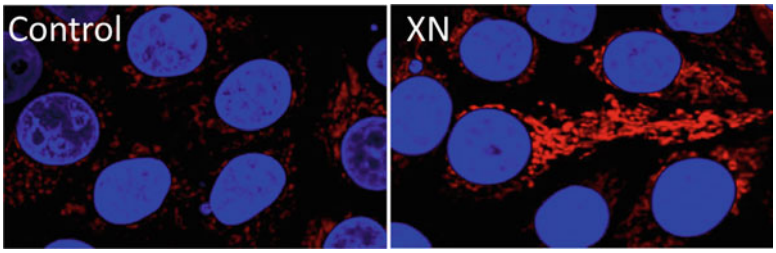

Fig. 4.4 Mitochondria are the source of XN-induced $O_2^{-\bullet}$. MCF-7 cells were loaded with MitoSOX Red and then incubated with 50 μM XN for 25 min. *Red* fluorescent staining of mitochondria indicates $O_2^{-\bullet}$ formation, blue staining depicts the position of nuclei. Pictures were acquired by confocal microscopy

with the antioxidants ascorbic acid and β-mercaptoethanol, which efficiently prevented XN-mediated apoptosis induction (Yang et al. 2007). 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-κB 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 (Monteghirfo et al. 2008).

Only recently, we could provide detailed information on the nature and source of ROS induced after XN treatment, and their link to apoptosis induction (Strathmann et al. 2010). 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 (Halliwell and Whiteman 2004). By using the dye dihydroethidium (DHE), which is specifically oxidized by $O_2^{-\bullet}$ (Zhao et al. 2005), we could demonstrate that XN treatment resulted in enhanced $O_2^{-\bullet}$-generation. $O_2^{-\bullet}$ 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 $O_2^{-\bullet}$, XN treatment caused a rapid increase in red fluorescent staining of mitochondria, suggesting that mitochondria are targeted by XN, leading to $O_2^{-\bullet}$-formation (Fig. 4.4). 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 $O_2^{-\bullet}$-generation induced by XN.

Blocking of mitochondrial respiration is believed to be one of the major sources of mitochondrial $O_2^{-\bullet}$ 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 $O_2^{-\bullet}$ formation is the initial trigger of XN-induced apoptosis (Strathmann et al. 2010).

4.4.4 ER Stress and Unfolded Protein Response

Besides the death receptor- and the mitochondria-mediated pathway of apoptosis induction, endoplasmatic 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 (Malhotra and Kaufman 2007). 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. 4.5).

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 (Zhang and Kaufman 2008). 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).

The UPR signaling cascades are activated by three ER membrane-localized sensor proteins: ATF6 (activating transcription factor 6), Ire1α (inositol-requiring 1α) 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.

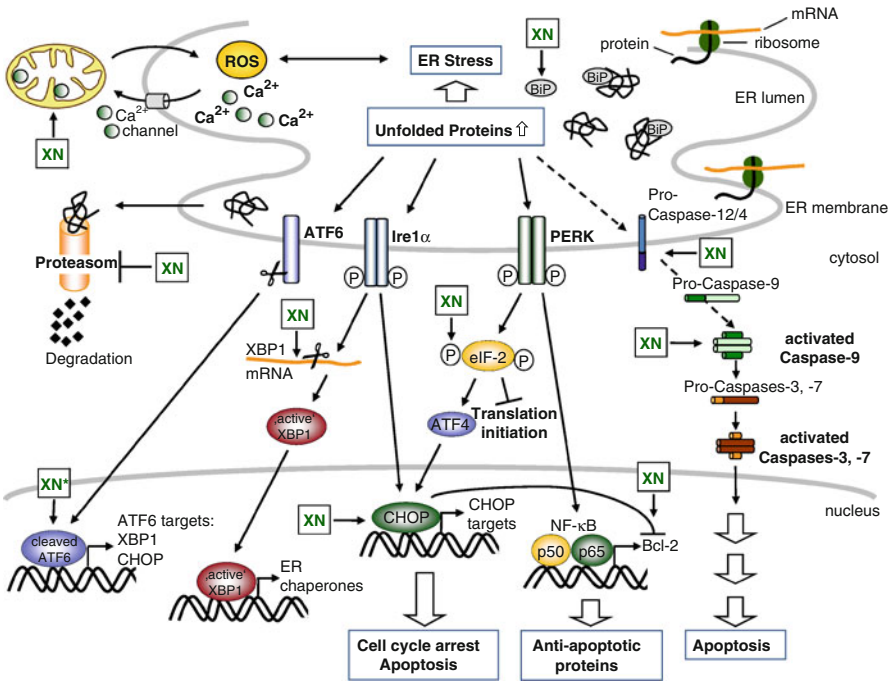

Fig. 4.5 Link between ER stress and unfolded protein response (*UPR*) and induction of apoptosis (Adapted from Faitova et al. 2006; Zhang and Kaufman 2008). Mechanisms targeted by XN in CLL cells are indicated (From Lust et al. 2009, *only in T47D cells used as control)

Activated PERK then phosphorylates the translation initiation factor eIF2α, 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-κB 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 Ire1α, 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 (Zhang and Kaufman 2008). 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 (Zhang and Kaufman 2008).

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 (Faitova et al. 2006).

Lust et al. have only recently provided first indication that XN induces apoptosis *via* an UPR-mediated mechanisms in CLL cells (Lust et al. 2009). The authors first demonstrated activation of caspases-3, -4, and -9 after XN treatment of CLL cells for 12 h and more prominently for 24 h. Next, they detected upregulation of the chaperone BiP (GRP78) at the mRNA and protein level. XN activated the ER stress sensor IRE1α, 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 eIF2α phosphorylation, which was strongly enhanced by XN treatment already after 3 h of treatment. Also, CHOP protein levels were strongly induced by XN treatment for 12 and 24 h. 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 observation are also relevant for other cell types and *in vivo*.

4.5 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 (Gerhauser 2005; Klenke 2008), 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 1,000 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% (Gerhauser 2005; Klenke 2008).

Albini et al. tested the effect of XN applied orally at a dose of 20 μM in drinking water on the growth of Karposi's sarcoma xenografts (Albini et al. 2006). 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. Twenty-four 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 (Albini et al. 2006).

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 μM 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-κB, phosphorylated IκB, and cytokine IL1β 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.

4.6 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-κB 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 $O_2^{-\bullet}$ 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 Karposi'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-κB activity.

Annex

Table 1 Anti-proliferative and apoptosis-inducing potential of XN *in vitro*

Organ	Cell lines	Xanthohumol concentration	Treatment time	Effect	Reference
Ovary	A-2780	0.1–100 μM	48, 96 h	↓ proliferation	Miranda et al. (1999)
Ovary	SK-OV-3	not stated	48 h	↓ proliferation	Lee et al. (2007)
Breast	MCF7	0. 1–100 μM	48, 96 h	↓ proliferation	Miranda et al. (1999)
		0.01–100 μM	8, 24, 48 h	Cytotoxicity at 100 μM	
		0.1–100 μM	24–96 h	↓ DNA synthesis	
		10, 40, 100 μM	8, 24 h	↔ induction of apoptosis	
Breast	MDA-MB-435	3–50 μM	72 h	↓ proliferation	Gerhauser et al. (2002)
		3–50 μM	72 h	↓ DNA synthesis	
		5–50 μM	24 h	S-phase cell cycle arrest	
Breast	MCF-7; T47D	1–20 μM	8 days	↓ proliferation	Vanhoecke et al. (2005)
	MCF-7	10 μM	96 h	↑ nuclear condensation	
	T47D	5–25 μM	48 h	↑ PARP cleavage	
Breast	Sk-Br-3	0.005–25 μM	72 h	↓ proliferation	Monteiro et al. (2007)
		0.005–25 μM	72 h	↓ DNA synthesis	
		5 μM	24 h	↓ proliferation	
		5 μM	24 h	↑ apoptosis	
Breast	MCF-7	10 μM	24 h	↓ viable cells	Guerreiro et al. (2007)
		10 μM	24 h	↓ proliferation	
		10 μM	24 h	↓ apoptosis	
Breast	MCF7	0.1–100 μM	24, 72 h	↓ viable cells; ↑ cytotoxicity	Monteiro et al. (2008)
		0.1–100 μM	24, 72 h	↓ DNA synthesis	
		10 μM	24 h	↓ proliferation	
Endometrium	Ishikawa	1.6–50 μM	72 h	↓ proliferation	Gerhauser et al. (2002)
Cervix	HeLa	not stated	72 h	↓ proliferation	Vogel and Heilmann (2008) and Vogel et al. (2008, 2010)

(contiuned)

Table 1 (continued)

Organ	Cell lines	Xanthohumol concentration	Treatment time	Effect	Reference
Prostate	BPH-1[a]/PC-3	2.5–20 μM	48 h	↓ proliferation	Colgate et al. (2007)
		10, 20 μM	48 h	↑ cell death	
		10, 20 μM	48 h	↑ caspase activity	
	BPH-1	10, 20 μM	48 h	S-phase cell cycle arrest	
		10, 20 μM	48 h	↓ NF-κB activity	
				↑ p65 nuclear translocation	
		10, 20 μM	48 h	↑ expression of Bax, p53	
				↓ Bcl-2 expression	
Prostate	PC-3, DU145	100, 200 μM	2 h	↓ cell viability	Delmulle et al. (2008)
		200 μM	up to 2 h	↔ caspase 3 activation	
Prostate	LNCaP	20–50 μM + TRAIL	48 h	↑ cytotoxicity in combination with TRAIL	Szliszka et al. (2010)
		20, 50 μM + TRAIL		synergistic ↑ apoptosis no necrotic cell death	
Prostate	BPH-1	1.6–50 μM	72 h	↓ proliferation	Strathmann et al. (2010)
		1.6–50 μM	5–45 min	↑ ROS production	
		3.1–50 μM	10–30 min	↓ Ψ_m	
		6.3–50 μM	30 min-24 h	↑ cytochrome *c* release	
		10–40 μM	48 h	↑ PARP cleavage	
		10–40 μM	72 h	↑ apoptosis (sub-G_1 fraction)	
Colon	HT-29	0.01–100 μM	48, 96 h	Cytotoxicity at 100 μM	Miranda et al. (1999)
Colon	HCT116 cl. 40-16	0.5–10 μM 5-15 μM	72 h 24-72 h	↓ proliferation ↑ PARP cleavage	Pan et al. (2005)
		5–15 μM	24–72 h	↑ caspase-3, -7, -8, -9 cleavage	
		5–15 μM	24–72 h	↓ Bcl-2 expression	
Colon	HCT15	not stated	48 h	↓ proliferation	Lee et al. (2007)
Liver	Primary rat hepatocytes	0.1–100 μM	24 h	Cytotoxicity at 100 μM	Miranda et al. (1999)
Liver	Hepa1c1c7	0.4–25 μM	48 h	↓ proliferation	Gerhauser et al. (2002)

Liver	AML12[a]; HA22T/VGH; HEP3B	10–225 μM	24 h	↓ proliferation	Ho et al. (2008)
	HA22T/VGH; HEP3B	90, 135 μM	4 h	↑ apoptosis	
		90 μM	24 h	↑ nuclear condensation	
		45, 90, 135 μM	24 h	↑ DNA fragmentation	
Liver	HSC[b]	0–40 μM	6 h	↑ caspase-3 activity	Dorn et al. (2010a)
		5, 10, 20 μM	24 h	↑ apoptosis/necrosis	
		5–40 μM	24 h	↓ vitality	
		5 μM	2 h	↓ basal and TNF-induced NF-κB activity	
	PHH[a]	25, 50 μM	24 h	↔ vitality	
		25, 50 μM	24 h	↔ apoptosis/necrosis	
		25, 50 μM	24 h	↓ basal and palmitate-induced pro-inflammatory IL-8 mRNA expression	
Liver	HepG2, Huh7	10–100 μM	24 h	↓ proliferation	Dorn et al. (2010b)
		25 μM		↑ caspase-3 activity	
	Huh7	2.5 μM	3 h preinc.	↓ TNF-induced NF-κB activity and IL-8 mRNA expression	
	PHH	10–100 μM	24 h	no inhibition of cell viability	
Lung	A549	not stated	48 h	↓ proliferation	Lee et al. (2007)
Leukemia	HL-60	0.5–10 μM	72 h	↓ proliferation	Gerhauser et al. (2002)
Leukemia	Primary B-CLL cells	10, 25 μM	24, 48 h	↑ cell death/apoptosis	Lust et al. (2005)
		25 μM	24, 48 h	↑ PARP cleavage	
Leukemia	MM6, U937	2.5–10 μM	24–72 h	↓ proliferation	Dell'Eva et al. (2007)
	primary AML and CLL cells	2.5–10 μM	24–72 h	↑ cell death	
		5 μM	6 h	↓ TNF-induced NF-κB signaling	
Leukemia	K562	2.5–10 μM	24–72 h	↓ proliferation	Monteghirfo et al. (2008)

(continued)

Table 1 (continued)

Organ	Cell lines	Xanthohumol concentration	Treatment time	Effect	Reference
	Primary CML cells	5 μM	24 h	↑ apoptosis	
		5 μM	6 h	↑ ROS, ↓ TNF-induced NF-κB signalling, ↑ p21, p53 mRNA and protein expression	
		5 μM	16, 24 h	↓ survivin mRNA and protein expression	
Leukemia	Primary CLL cells	25 μM	12, 24 h	↑ caspase-3, -4, -9 cleavage	Lust et al. (2009)
		25 μM	3–24 h	↓ Bid, Mcl-1 expression	
		25 μM	24 h	↓ Bcl-2 expression	
		25 μM	12, 24 h	↑ ER-stress, ↑ UPR	
		25 μM	12 h	↑ ROS	
		25–100 μM		↓ S20 proteasomal activity	
		25 μM	12 h	↑ ubiquitinated proteins	
Leukemia	KBM-5 (CML)	50 μM	4 h	↑ TNF-induced apoptosis	Harikumar et al. (2009)
		50 μM	12, 24 h	↑ TNF-induced PARP cleavage	
		50 μM	4 h + 0–12 h	↓ expression of TNF-induced proliferative and anti-apoptotic proteins	
		50 μM/5–50 μM	1–12 h/4 h	↓ TNF-induced activation of NF-κB, IκBα kinase activity	
Myeloma	U266	50 μM	4 h	↑ TNF-induced apoptosis	Harikumar et al. (2009)
Sarcoma	HT-1080	3 μM	72 h	↓ proliferation under hypoxia	Goto et al. (2005)
Sarcoma	KS-IMM	2.5–25 μM	48–72 h	↓ proliferation	Albini et al. (2006)
Melanoma	SK-Mel2	not stated	48 h	↓ proliferation	Lee et al. (2007)
Macrophages	Raw264.7	0.4–50 μM	24 h	↔ proliferation	Gerhauser et al. (2002)
Endothelial cells	HUVEC	2.5–25 μM	48–72 h	↓ proliferation (10–15 μM); ↑ cell death (25 μM)	Albini et al. (2006)
		10 μM	3 h pre-treatment	↓ TNF-induced NF-κB nuclear translocation, ↓ IκBα phosphorylation	

Endothelial cells cells	HUVEC	2.5-10 μM	24-72 h	↓ proliferation	Dell'Eva et al. (2007)
Adipocytes	From 3 T3-L1	25–100 μM	24, 48 h	↓ proliferation	Yang et al. (2007; 2008); Rayalam et al. (2009)
		25–100 μM	24, 48 h	↑ apoptosis	
		75 μM	0–3 h	↑ ROS production	
		75 μM	0–1.5 h	↓ mitochondrial membrane potential	
		75 μM	6–48 h	↑ cytochrome *c* release	
		75, 100 μM	3–12 h	↑ caspase3/7 activity	
		75 μM	6–24 h	↑ PARP cleavage	
Adipocytes	From 3 T3-L1	0.1–50 μM	24–72 h	↓ proliferation	Mendes et al. (2008)
		5 μM	24 h	↓ proliferation	
		5 μM	24 h	↑ apoptosis	
Dendritic cells	From bone marrow	2–50 μM	24 h	↑ caspase-3, -8 activity	Xuan et al. (2010)
		20 μM	24 h	↑ caspase-3, -8 cleavage	
		2–50 μM	24 h	↑ apoptosis (sub-G_1 fraction)	
T-cells	Murine T-lymphocytes	1.25–40 μM	72, 96 h	↓ Con A or IL-2-induced proliferation	(Gao et al. 2009)
		1.25–40 μM	72 h	↓ cell viability at high, ↑ cell counts at low concentrations	

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

[b]*HSC* hepatic stellate cells

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