Xanthohumol Activates the Proapoptotic Arm of the Unfolded Protein Response in Chronic Lymphocytic Leukemia

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Abstract. Background: Chronic lymphocytic leukemia (CLL) is an incurable disease with a natural history of increasing resistance to chemotherapy. A novel approach to overcome chemotherapy resistance may be targeting the endoplasmic reticulum (ER). Patients and Methods: The involvement of the unfolded protein response (UPR) in the cell killing effect of xanthohumol (X) was examined in 18 patient samples. Results: X-induced apoptosis of CLL cells was accompanied by the induction of glucose-regulated protein of 78 kDa (GRP78) and heat-shock protein of 70 kDa (Hsp70) protein levels and by sustained phosphorylation of the eukaryotic translation initiation factor 2 (eIF2α), suggesting the involvement of the ER stress transducer, the double-stranded RNA-activated protein kinase (PKR)-like ER kinase (PERK). The X-box-binding protein 1 (XBP1) mRNA was spliced but no clear activation of activating transcription factor 6 (ATF6) was observed. The proapoptotic outcome was further demonstrated by the up-regulation of CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP), down-regulation of myeloid cell leukemia 1 (Mcl-1) and B-cell lymphoma 2 (Bcl-2), cleavage of poly-(ADP)-ribose polymerase (PARP) and processing of caspase-3, -4 and -9. Furthermore, X showed proteasome inhibitory activity. Conclusion: X stimulates the proapoptotic arm of the UPR in ex vivo CLL cells, suggesting that ER stress may play an important role during X-induced apoptosis.

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Chronic lymphocytic leukemia (CLL) is the most common form of leukemia in the Western world. The clinical progression of CLL results from the gradual accumulation of mature, non-dividing B-lymphocytes (predominantly in the G0-phase of the cell cycle) in the peripheral blood, lymph nodes, spleen and bone marrow (1). In comparison to normal B lymphocytes, CLL cells appear to have a more extensive endoplasmic reticulum (ER). As such, this organelle may represent an attractive therapeutic target (2). Considering that CLL cells, like many other malignant cells, develop resistance to chemotherapy with treatments that activate the mitochondrial death machinery, agents capable of initiating apoptosis at other organelles, such as the ER, may have therapeutic potential. Killing malignant CLL cells by targeting the ER, has been shown to overcome fludarabine resistance in vitro (3). The recent observation that proteasome inhibitors (such as bortezomib) have been found to induce ER stress (4-5), make it reasonable to assume that manipulation of the ER may enhance the efficacy of chemotherapeutic drugs and provide new anticancer targets. Preclinical data support the potential of proteasome inhibition for CLL in vitro, although monotherapy has been disappointing in clinical trials (6-7).

The ER is the principal site of protein folding, synthesis and modification of secretory, cell-surface and secretory pathway proteins. Its proper functioning is essential for the cell and mechanisms have evolved to cope with disturbances of these functions which result in ER stress. One of these mechanisms is the unfolded protein response (UPR) which is activated when protein folding is disrupted and unfolded or misfolded proteins are accumulating in the ER (8). Under ER stress, the protein levels of ER-resident stress proteins and molecular chaperones referred to as glucose-regulated proteins (GRPs) or heat-shock proteins (Hsps) are elevated (9). The central player in the UPR is the ER-resident...
chaperone GRP78, which controls the ER stress transducers, namely activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1) and double-stranded RNA-activated protein kinase (PKR)-like ER kinase (PERK). The UPR is mainly a self-protective mechanism, however, prolonged ER stress eventually leads to activation of the cell death program. Indeed, evidence is emerging that the ER, like the mitochondria, can directly initiate death signals or sensitize mitochondria to a variety of extrinsic or intrinsic death signals and in this way represents a third pathway to apoptosis (8). A number of pharmacological agents that directly or indirectly induce ER stress have been shown to kill CLL cells. Brefeldin A, an inhibitor of ER-Golgi protein transport, has been shown to induce apoptosis in CLL cells accompanied by abnormal ER swelling and to kill fludarabine-refractory CLL cells (3). Tetrocarin A, an antibiotic agent isolated from actinomycetes, induces apoptosis via ER stress accompanied by the up-regulation of Hsp70 (10). Proteasome inhibitors such as bortezomib induce ER stress in myeloma cell lines (4) and have been documented to stimulate apoptosis in CLL in vitro (11).

In recent years plant-derived flavonoids, such as the prenylated chalcone xanthohumol (X), have been characterized as broad-spectrum cancer chemopreventive agents (12). X has been linked to diverse anticancer effects, such as reduced cell proliferation, angiogenesis and invasion (13-14). Some of these effects have been linked to nuclear factor-kappaB (NF-κB) inhibition because of the indirect effects of X on downstream targets in prostate epithelial cells (15) and breakpoint cluster region-abelson murine leukemia (Bcr-Abl)-positive leukemia cells (16). Reported data on inhibitory doses are contradictory, however, recent data have shown a direct inhibitory effect on IkB kinase (IKK) starting from 50 μM X (17). Previous research in our laboratory has shown that X induced a dose- and time-dependent killing of CLL cells at an LD50 (24 h) of 24.4±6.6 μM. Cell death was accompanied by cleavage of poly-(ADP)-ribose polymerase (PARP), a caspase-3 substrate, in a dose range below that which exerts the effects on NF-κB (18). In the present study, the molecular effects of a sublethal concentration of X (25 μM) on the UPR in vitro as a proapoptotic strategy in CLL were explored.

**Patients and Methods**

**Chemicals.** X was kindly provided by Prof. D. De Keukeleire (Laboratory of Pharmacognosy and Phytochemistry, Ghent University, Belgium). X (stock: 25 mM) was dissolved in ethanol (EtOH) (Merck, Darmstadt, Germany); brefeldin A (stock: 500 μg/ml) (Sigma, Bornem, Belgium), MG132 (stock: 1 mM) (Calbiochem, EMD Biosciences, San Diego, CA, USA), phorbol-12-myristate-13-acetate (PMA, stock: 1 mg/ml) (Sigma), the pancaspase inhibitor Z-VAD-fmk (stock: 1 mg/ml) (Sigma), the pancaspase inhibitor Z-VAD-fmk (stock: 10 mM) (BD Pharmingen, Erembodegem, Belgium) in dimethylsulfoxide (DMSO) (Sigma) and bortezomib (stock: 10 μM) (Johnson and Johnson, Beerse, Belgium) in distilled water.

**Patient characteristics, CLL lymphocyte isolation and cell culture.** Peripheral blood samples were obtained from 18 CLL patients after informed consent according to the institutional guidelines. The patient characteristics are summarized in Table I. The mononuclear cell layer (PBMCs) was separated by using Ficoll-Hypaque density gradient centrifugation of the whole blood (Lymphoprep, Nycomed Pharma Inc., Marlborough, MA, USA).

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### Table I. Clinical characteristics of CLL patients.

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FCR: Fludarabine, Cyclophosphamide and Rituximab; M: mutated; Na: not available; U: unmutated; UPN: unique patient number; ZAP70: zeta-associated protein of 70 kDa.
AS, Oslo, Norway). The cells were resuspended in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Greiner Bio-one, Wemmel, Belgium), 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.56 μg/ml fungizone (Gibco BRL, Merelbeke, Belgium). CD19-positive cells were selected by magnetic nanoparticle positive selection (EasySep Human CD19-positive Selection Kit, Stem Cell Technologies, Vancouver, BC, Canada) to a purity of 99% CD19-positive B-cells. The mammary adenocarcinoma cell line T4-D-7 was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM (Invitrogen, Merelbeke, Belgium) supplemented with 10% heat-inactivated FBS (Greiner Bio-one), 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.56 μg/ml fungizone 2.5 μg/ml (Gibco BRL).

**Measurement of apoptosis by flow cytometry.** Cell death in the CLL cells was assessed by flow cytometry (Cytomics FC500 1 laser, Beckman Coulter, Fullerton, CA, USA) as previously described (Human AnnexinV–FITC (AnnV) – Propidium Iodide (PI) Detection Kit, Bender MedSystems Diagnostics, Vienna, Austria) (18). All the measurements were made in duplicate and averaged.

**Fluorescence microscopy and staining for reactive oxygen species (ROS).** X- and EtOH-treated (control) CLL cells were washed once with ice-cold PBS. After centrifugation, the cells were harvested in 1 ml PBS and stained with 1 μM 2',7'-dichlorodihydrofluorescein diacetate (DCFDA, stock: 20 mM) (Sigma) for 10 min at 37˚C. After staining, the cells were visualized by a Carl Zeiss Axio Vision fluorescence microscope (Carl Zeiss, Göttingen, Germany).

**Electrophoretic mobility-shift assay (EMSA).** After appropriate induction, the CLL cells were washed with ice-cold PBS and precipitated by centrifugation for 15 min at 1500 rpm (4˚C). The preparation of the total cellular extracts has been described previously (19). Equal amounts of protein were incubated for 25 min with a NF-κB-specific 32P-labeled oligonucleotide and binding mix, as described previously (20-21). The labeling of the oligonucleotides was performed with [α-32P]-dCTP by using Klenow enzyme (Boehringer Mannheim, Mannheim, Germany). The NF-κB oligonucleotide comprises the sequence 5'-AGCTATGTGGGTTTT (Boehringer Mannheim, Mannheim, Germany). The CD19-positive cells were selected by magnetic nanoparticle positive selection (EasySep Human CD19-positive Selection Kit, Stem Cell Technologies, Vancouver, BC, Canada) to a purity of 99% CD19-positive B-cells. The mammary adenocarcinoma cell line T4-D-7 was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM (Invitrogen, Merelbeke, Belgium) supplemented with 10% heat-inactivated FBS (Greiner Bio-one), 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.56 μg/ml fungizone 2.5 μg/ml (Gibco BRL). The NF-κB-specific 32P-labeled oligonucleotide and binding mix, as described previously (20-21). The labeling of the oligonucleotides was performed with [α-32P]-dCTP by using Klenow enzyme (Boehringer Mannheim, Mannheim, Germany). The NF-κB oligonucleotide comprised the sequence 5'-AGCTATGTGGGTTTT GCCATGACC-3' in which the single interleukin 6 (IL-6) promoter-derived NF-κB motif is underlined. The samples were loaded on a 6% polyacrylamide gel and run in 0.5 x TBE buffer (pH=8). The gel was dried after electrophoresis and the complexes formed were analysed and quantified using Phosphor Imager Technology (Molecular Dynamics, GE Healthcare, Diegem, Belgium).

**Western blotting.** After treatment for different times, cell lysates were prepared and immunostaining of the blots was performed as previously described (18). Proteins were separated on a 10% or 16% SDS-polyacrylamide gel. The primary antibodies used were anti-B-cell lymphoma 2 (Bcl-2) homologous antagonist/killer (Bak), anti-caspase-7 and anti-caspase-8 from BD Pharmingen (BD Biosciences, San Diego, CA, USA); anti-ATF6α, anti-eukaryotic translation initiation factor 2 (eIF2α) and anti-phospho-eIF2α from Cell Signaling Technology (Beverly, MA, USA); anti-ATF6α, anti-eukaryotic translation initiation factor 2 (eIF2α) and anti-phospho-eIF2α from Biosource (Camarillo, CA, USA); anti-cleaved caspase-3 (Asp175) from Cell Signaling Technology (Beverly, MA, USA); anti-Bcl-2 homology 3 (BH3)-interacting domain death agonist (Bid) from R and D Systems (ImmunoSource, Halle, Belgium); anti-Bcl-2-associated X protein (Bax), anti-Bcl-2-like 1 (Bcl-xL), anti-growth arrest- and DNA damage-inducible gene 153 (GADD153, also known as CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP)), anti-GRP78, anti-heat-shock protein of 90 kDa (Hsp90), anti-myeloid cell leukemia 1 (Mcl-1) and anti-ubiquitin (Ub) (P4D1) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-α-tubulin from Sigma; anti-ATF6 from Stratagene (La Jolla, CA, USA); anti-caspase-4, anti-caspase-9, anti-GRP94 and anti-Hsp70 from Stressgen (Victoria, BC, Canada). Quantification of the autoradiograms was conducted using Quantity One software (Bio-Rad, Eke, Belgium).

**PCR amplification and real-time quantitative RT-PCR.** The CD19 selected cells were washed once with ice-cold PBS after treatment and quickly stored at −80˚C until use. The total RNA was extracted from the CLL cells using an RNeasy Mini Kit (Qiagen, Venlo, the Netherlands) according to the manufacturer’s instructions. The total RNA (2 μg) was treated with RQ1 RNase-free DNase from Promega (Leiden, the Netherlands). First-strand cDNA was synthesized using an iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer’s instructions. In order to quantify the gene expression level of heat-shock 70 kDa protein 5 (HSPA5, known as GRP78), primer sequences were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA) and were placed on different exons. The primer sequences are available from the Real-Time PCR Primer and Probe Database (gene: primer-ID: HSPA5: 3477) (22). Relative gene expression levels were determined using a SYBR Green I RT-PCR assay as described by Vandesompele et al. (23) and the comparative cycle time (Ct) method (also called ΔCt method) was used for quantification. The PCR reagents were obtained from Eurogentec (Seraing, Belgium) as SYBR Green I mastermixes and used according to the manufacturer’s instructions. The PCR reactions were run on an ABI prism 7000 Sequence Detection System (Applied Biosystems). To correct for differences in the RNA quantities and cDNA synthesis efficiency, relative gene expression levels were normalized using the geometric mean of three housekeeping genes (ribosomal protein L13a (RPL13A), ubiquitin C (UBC) and hypoxanthine phosphoribosyltransferase 1 (HPRT1)) according to Vandesompele et al. (24). The normalized expression level (%) of GRP78 of each treated patient sample was compared with that of its corresponding EtOH-treated sample (solvent control) at a particular time-point. In order to amplify the fragments coding for the unsliced X-box binding 1 (XBP1(u)) and spliced XBP1 (XBP1(s)) the primers 5'-TGTTGTGCTGAAGAGGGAGCGCAG-3' and 5'-GAAAGG GACCGCTGTTAGGAC-3' were used (25). The relative gene expression levels were normalized using the housekeeping gene actin. The actin primers were 5'GGGGAGGAATGTCTTCTGTA-3' and 5'GGAGATATCTTCAACTGACTG-3'. The PCR reactions were performed in a 1x TaKaRa ExTaqTM PCR reaction mixture (TaKaRa Bio Inc., Shiga, Japan) according to the manufacturer’s instructions. The PCR amplifications were performed for 35 cycles on a PTC-0150 Miniicycler (Bio-Rad) using the following cycling parameters: 95˚C for 10 sec, 60˚C for 30 sec, and 72˚C for 1 min with a final extension period of 72˚C for 10 min. The samples were loaded on a 3% agarose gel.

**Assessment of the 20S proteasomal activity.** The 20S proteasome forms the proteolytic core of the 26S proteasome. The measurement of the 20S proteasome activity was based on the chymotryptic activity of the proteasome and was performed as described by Elliott et al. (26-27). Samples of human pooled whole blood were incubated with bortezomib, EtOH and X for 1 hour at 37˚C. After incubation, lysates were prepared by adding 5 mM EDTA. After...
centrifugation, the supernatant was stabilized by adding stabilizing buffer (40 mM HEPES (pH 8.0), 1 mM EDTA, 20% glycerol). The calibration of the fluorometer was established by means of a standard curve with 7-amino-4-methylcoumarin (AMC, Sigma). The standards were prepared in chymotryptic substrate buffer (26 μM HEPES (pH 8.0), 0.7 mM EDTA, 0.065% SDS, 78 μM chymotryptic substrate (Suc-Leu-Leu-Val-Tyr-AMC, Bachem Bioscience, King of Prussia, PA, USA). Fluorescence was measured using an excitation wavelength of 380 nm and an emission wavelength of 450 nm on a Safire2 plate reader (Tecan Benelux, Mechelen, Belgium). The activity was normalized to the amount of protein present in each sample.

Data analysis. Data are presented as the mean±SD (standard deviation) for different experiments. Statistical analysis of the data between treated and solvent control group was performed with Student’s t-test and a p-value less than 0.1 was considered significant.

Results

Effect of X on caspase-dependent and -independent pathways.

A panel of caspases was investigated in a group of 14 CLL patients samples after 3, 6, 12 or 24 h of treatment with 25 μM X or 0.1% EtOH solvent control E. Western blot evaluation using tubulin as loading control and mean relative intensity (%) assessed by quantification of the radiograms. (A-B) Caspase-3 (n=8), -4 (n=4), -7 (n=4), -8 (n=4) and -9 (n=8). (C-D) Mcl-1 (n=9), Bcl-xL (n=7), Bak (n=7), Bax (n=8) and Bid (n=6). (E) CLL cells stained with Bcl-2-FITC after 24 h treatment and evaluated by flow cytometry. (F) Relative percentage living cells of CLL cells treated with 25 μM X or 0.1% EtOH, E, and pretreated with 20 μM or 50 μM Z-VAD-fmk (Z) after 24 or 48 h incubation time. Error bars: standard deviation, *p<0.05.
(FL1 channel) and the mean fluorescence intensity (MFI) of the X-treated cells (49.14) was down-regulated after 24 h compared to the control cells (79.15), as demonstrated in Figure 1E. In order to evaluate if X-induced cell death was solely dependent on the processing of caspases, the pan-caspase inhibitor Z-VAD-fmk was used. However, at the dose administered (20 and 50 μM), Z-VAD-fmk exerted no effect on the percentage of dead cells induced by X (Figure 1F), suggesting that other pathways may also be implicated in X-induced apoptosis in CLL.

Effect of X on the ER stress-related chaperones GRP78 and Hsp70. X (25 μM) elevated the expression levels of GRP78 mRNA from 3 h of treatment (Figure 2A). The increased levels of GRP78 were confirmed by Western blotting and furthermore, the protein levels of the chaperone Hsp70 were up-regulated after 12 h treatment of the CLL cells with X. Brefeldin A was used as positive control and clearly up-regulated both GRP78 and Hsp70 in the CLL patient samples. In contrast to brefeldin A, the protein levels of Hsp90 and GRP94 were not affected by X (Figure 2B and 2C).

Involvement of the ER stress transducers IRE1, ATF6 and PERK in X-induced apoptosis. The activation of IRE1 was indirectly analyzed via the detection of XBP1(s). Both X- and EtOH-treated CLL cells expressed XBP1(u) mRNA while XBP1(s) mRNA could be detected after 24 h treatment with X (Figure 3A and 3B). However, no clear band of 54 kDa, representing the active XBP1(s), could be detected by Western blotting after different treatment times. In the positive control, brefeldin A-treated cells, a clear XBP1(s) mRNA band was demonstrated as well as protein (data not shown). Immunostaining of ATF6 showed different patterns amongst different patients with different ATF6 antibodies. Generally, the total ATF6 (p90ATF6) demonstrated both non- and glycosylated forms but different bands appeared between 50 and 75 kDa. However, no clear cleavage band of 50 kDa (reported MW of cleaved ATF6) was detected (n=6), whereas a strong band at 50 kDa was visualized in X-treated T47-D cells which were used as positive control. The involvement of PERK was indirectly examined by measuring the levels of phosphorylated eIF2α. X strongly enhanced the phosphorylation levels of eIF2α as early as 3 h treatment, persisting until 24 h, whereas the total eIF2α levels remained unchanged (Figure 3C and 3D). The protein level of CHOP downstream of PERK was analyzed by Western blotting. Figure 3E and 3F show that X elevated the CHOP levels from 12 h on. These results were confirmed with the positive control brefeldin A. An accumulation of ROS was also clearly demonstrated in the presence of X from 6 h which disappeared after 24 h, as visualized by fluorescence microscopy (Figure 3G).

Proteasome inhibitory characteristics. X induced the accumulation of polyubiquitinated proteins, indicative of proteasome inhibition after 6 h of treatment, but this was
more apparent after 12 h (as demonstrated with MG132 and bortezomib) (Figure 4A and 4B). Furthermore, X inhibited the 20S proteasomal activity at concentrations of 25 μM and above (Figure 4C).

**NF-κB inhibitory features.** The dose of 25 μM X showing cytotoxicity in our experiments induced no significant inhibition of untreated or PMA (100 ng/ml)-stimulated NF-κB DNA-binding of p50-p65 NF-κB heterodimer by EMSA (Figure 5A). PMA-stimulated NF-κB activation (used as positive control) was associated with high phosphorylation levels of IKK and a decrease of inhibitor of NF-κB alpha (IκBα), as expected. After 1 h, IκBα was resynthesized. In contrast, X exposure was not associated with changes in IKK phosphorylation while total cytoplasmic IκBα protein levels even decreased after 60 min treatment (Figure 5B and 5C).

**Discussion**

X-induced cell death in CLL was both caspase-dependent and -independent as previously described in prostate cancer cells (10). Other cell death mechanisms were therefore explored with an emphasis on ER stress. Based upon our previous observations of the CLL cell killing effect of X (18), the present report provides a mechanistic basis of the potent anti-CLL activity of X, showing that X stimulated the proapoptotic arm of the UPR as a result of prolonged ER stress, and failed to stimulate the pro-survival arm. As demonstrated with X in CLL, also other flavonoids are able to activate PERK in human leukemia cells (M1, P388, U-937) (28). Activation of this arm of the UPR results in a general protein synthesis block, allowing cells to cope with the protein overload and may activate the antiapoptotic NF-κB (29-30). In contrast to
previous reports (15-17), the NF-κB inhibitory activity of X in CLL could not be confirmed at the concentration (25 μM) used in the present study. In addition to PERK, the ER stress transducer IRE1, but not ATF6, was involved in the X-induced apoptosis. Surprisingly, the transient increase of XBP1(s) was not translated into a corresponding increased protein expression. This may suggest incapacity of the CLL cells to translate the XBP1(s) transcript. On the other hand, protein levels of XBP1(s) may have been too low to be detected by Western blotting in the CLL cells due to lability of the XBP1 mRNA or the short lifetime of the XBP1(s) protein (31). However, the specific role of XBP1 in CLL remains to be elucidated. Apart from translation block, activation of PERK may up-regulate the expression levels of proapoptotic CHOP via selective translation of ATF4 (29). In turn, CHOP suppresses Bcl-2 expression and induces ROS accumulation (32). The terminal UPR induced by X was confirmed by the increased CHOP levels and cleavage of the ER membrane-resident caspase-4, which processed the further downstream caspase-9 and -3.

Given the combined evidence from the literature of NF-κB inhibition by X at higher doses and our observations of ER stress induction in the lower dose range, the capability of X as a potential proteasome inhibitor was investigated. Since proteasome inhibitors induce ER stress and NF-κB inhibition, through the accumulation of polyubiquitinated proteins such as IkBα (33), Western blot analysis of protein ubiquitination was included in our search for a potential primary target. The accumulation of polyubiquitinated proteins in CLL and the inhibition of 20S proteasomal activity by X was demonstrated. Proteasome inhibitors may function as ER stress inducers and as activators of the UPR (5, 34). They are attractive therapeutic agents for various malignancies and their anticancer activity is mediated by diminished proliferation, cell death induction and the down-regulation of chemoresistance (35). The proteasome inhibitor bortezomib, which is able to activate the UPR in plasma cells (4, 5), has revolutionized the treatment of multiple myeloma (36). Furthermore, CLL cells are characterized by a constitutive altered ubiquitin-proteasome system which could play a central role in inducing apoptosis (37). The proteasome inhibitory properties of X are in agreement with other reports of polyphenolic compounds, such as (−)-epigallocatechin-3-gallate (EGCG) and resveratrol, which interfered with proteasome activity and were able to induce ER stress (38-40).
In conclusion, X-induced ER stress and apoptosis by the activation of the proapoptotic arm of the UPR in CLL cells may be secondary to inhibition of the proteasome. X represents a prospective cytotoxic agent for the treatment of CLL.

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