



Proteasome inhibitor-induced apoptosis of B-chronic lymphocytic leukaemia cells involves cytochrome c release and caspase activation, accompanied by formation of an ~700 kDa Apaf-1 containing apoptosome complex

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Proteasome inhibitors, including lactacystin and MG132 (carbobenzoxyl-leucinyll-leucinyll-leucinal), potently induce apoptosis in leukaemic B cells from patients with B cell lymphocytic leukaemia (B-CLL). This pro-apoptotic effect occurs in cells from patients at all stages of the disease, including those resistant to conventional chemotherapy, suggesting that proteasome inhibitors may be useful for treatment of B-CLL. Following initial inhibition of proteasomal activity, these agents induce mitochondrial cytochrome c release and caspase-dependent apoptosis, involving cleavage/activation of caspases -2, -3, -7, -8 and -9. Pre-treatment with the cell permeable caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp (OMe)fluoromethyl ketone (Z-VAD.fmk), did not prevent the release of cytochrome c or partial processing of caspase-9 but prevented activation of effector caspases and the induction of apoptosis. These results suggest that the release of cytochrome c is caspase independent and that caspase-9 is the initiator caspase in proteasome inhibitor-induced apoptosis of B-CLL cells. Activation of B-CLL lysates with dATP results in the formation of an ~700 kDa caspase-activating apoptosome complex containing Apaf-1. We describe for the first time the formation of a similar ~700 kDa caspase-activating apoptosome complex in B-CLL cells induced to undergo apoptosis by proteasome inhibitors. *Leukemia* (2001) 15, 1388–1397.

Keywords: apoptosis; B-CLL; apoptosome; proteasome inhibitors; caspases

Introduction

B cell chronic lymphocytic leukaemia (B-CLL) is the most common leukaemia worldwide but its aetiology is unknown.^{1,2} This leukaemia is identified by the accumulation of immunologically immature non-proliferating cells, which co-express CD5 and CD23 as well as the pan B cell markers CD19, CD20 and CD40.^{1,3} The malignancy is characterized by the failure of cells to undergo apoptosis rather than excessive cellular proliferation, resulting in the gradual accumulation of leukaemic B cells.³ Drug resistance presents a great obstacle to the treatment of B-CLL and complete remission of the disease is uncommon. However, recent work has demonstrated that proteasome inhibitors potently induce apoptosis in B-CLL cells *in vitro*. MG132 induced apoptosis in glucocorticoid-resistant B-CLL cells,⁴ and lactacystin activated apoptotic cell death and sensitised resistant B-CLL cells to TNF α -induced apoptosis.^{5,6}

Caspases, a family of aspartate-specific cysteine proteases, are responsible for many of the characteristic biochemical and morphological changes associated with the execution phase of apoptosis.^{7,8} 'Initiator' caspases with long prodomains such as caspases-8, -9 and -10 either directly or indirectly activate 'effector' caspases, such as caspases-3, -6 and -7, which have

short prodomains. These effector caspases then cleave intracellular substrates such as poly (ADP-ribose) polymerase and lamins during the execution phase of apoptosis.^{7,8} Caspase-8 is the initiator caspase in death receptor-mediated apoptosis, such as CD95 (Fas/APO-1), whereas caspase-9 is the most upstream caspase in chemical-induced apoptosis.^{9–13} Chemical-induced apoptosis results in perturbation of mitochondria followed by release of mitochondrial cytochrome c, which binds to Apaf-1.^{11,12,14,15} Apaf-1, the first mammalian CED-4 homologue, is an ~130 kDa protein which contains an N-terminal caspase recruitment domain (CARD) and a C-terminal domain containing WD40 repeats involved in protein-protein interactions.¹² Following binding of cytochrome c, dATP/ATP binds to the CED-4 homology domain of Apaf-1 resulting in its oligomerisation. The CARD domain at the N-terminus of caspase-9 then binds to the CARD of Apaf-1 resulting in recruitment and activation of caspase-9, which subsequently activates the effector caspases-3 and -7, thereby initiating a caspase cascade that effectively dismantles the cell. This Apaf-1 containing complex has been referred to as the apoptosome.^{16–18} Following dATP-dependent activation of lysates, we have previously described the formation of an ~700 kDa biologically active caspase processing complex and a larger ~1.4 MDa biologically inactive apoptosome.¹⁹ Additionally, we showed predominant formation of the ~700 kDa active complex in cells undergoing chemical-induced apoptosis.

Caspase-2, -3, -7 and -8 are present in untreated B-CLL cells as their unprocessed zymogens and the effector caspases -3 and -7 are activated following the induction of apoptosis by the glucocorticoid, prednisolone or the alkylating agent chlorambucil, two agents commonly used in the treatment of CLL.^{20–23}

Although caspases play a key role in the execution phase of apoptosis, much less is known about the role of the proteasome in apoptosis. The 26 S proteasome, the major extra-lysosomal machinery for protein degradation, is responsible for the ATP-dependent degradation of proteins targeted for destruction by ubiquitin.²⁴ These include short-lived regulatory proteins critical for cell proliferation and cell cycle regulation such as the tumor suppressor p53, various cyclins and the cyclin-dependent kinase inhibitor p27^{Kip1}.²⁵ The proteasome is also important for the processing and degradation of NF- κ B and I κ B and for the proteolysis of other transcription factors including c-fos and c-jun.²⁶ Proteasomal activity appears essential for the survival of proliferating cells as treatment with proteasome inhibitors results in apoptosis, which is most probably due to the accumulation of short-lived regulatory proteins such as p53, p27^{Kip1} and c-myc.^{26–31} In contrast treatment of quiescent or terminally differentiated cells, such as thymocytes or neuronal cells, blocks cell death induced by diverse agents^{32,33} suggesting that they may act by blocking the proteasome-mediated proteolysis of a caspase inhibitor.²⁵ Taken together these data support the hypothesis that the pro-

teasome acts upstream of caspases and may be a therapeutic target in certain malignancies. A potential anti-cancer role for proteasome inhibitors has previously been described, showing that they can induce apoptosis in human prostate carcinoma cells independent of functional Bcl-2 and p53³⁴ and that they can inhibit tumour growth.^{35,36} Inhibition of NF- κ B activity by proteasome inhibitors has been proposed as a mechanism for induction of apoptosis in B-CLL cells,^{4,5} however, in a recent study no alterations of nuclear NF- κ B, or cytoplasmic I κ B α , were observed after treatment with lactacystin.⁶

In this study we investigated the execution mechanisms involved in proteasome inhibitor-induced apoptosis of B-CLL cells. We show proteasomal inhibitors induce apoptosis by formation of an ~700 kDa caspase-activating apoptosome complex containing Apaf-1, thus initiating a post-mitochondrial caspase cascade with caspase-9 as the initiator caspase.

Materials and methods

Materials

Lactacystin was from Affiniti Research Products (Exeter, UK) and MG132 (carbobenzoxy-leuciny-leuciny-leucinal) from Calbiochem (La Jolla, CA, USA). LLnL (N-acetyl-N-leuciny-L-leuciny-L-norleucinal, calpain inhibitor I), prednisolone, chlorambucil, propidium iodide and Hoechst 33342 were from Sigma (Poole, UK). Annexin V-FITC was from Bender MedSystems (Vienna, Austria) and DiOC₆(3) (3,3' dihexyloxycarbocyanine iodide) was from Molecular Probes (Eugene, OR, USA). Benzyloxycarbonyl-Val-Ala-Asp (OMe)fluoromethyl ketone (Z-VAD.fmk) and benzyloxycarbonyl-Asp-Glu-Val-Asp aminofluoromethyl coumarin (Z-DEVD.AFC) were from Enzyme Systems (Dublin, CA, USA).

B cell purification and culture

B-CLL patients, who had not received prior chemotherapy, were staged according to the Binet system.³⁷ Peripheral blood samples, obtained after informed consent and with local ethical committee approval, were purified as previously described²³ except that T cells were removed using anti-CD3 Dynabeads (Dyna, Merseyside, UK). B-CLL cell purity was assessed by staining with anti-CD19/FITC and anti-CD5/RPE antibodies (Dako, Cambridge, UK) and analysed by flow cytometry. This method produced an ~95% pure population of cells expressing both CD19 and CD5. Purified B-CLL cells were re-suspended in RPMI 1640 medium at a density of 1 to 4 \times 10⁶/ml and incubated at 37°C in an atmosphere of 5% CO₂ with prednisolone (20 μ M), chlorambucil (10 μ M) or with the proteasome inhibitors, lactacystin (0.625–10 μ M), MG132 (0.125–1 μ M) or LLnL (1.25–10.0 μ M). At the indicated times, cells were analysed for apoptosis, lysates were prepared, or cell pellets stored at –80°C for subsequent Western blotting.

Quantification of apoptosis

Apoptotic cells were quantified by an increase in externalised phosphatidylserine as assessed by Annexin V binding.^{23,38} Apoptotic cells, with a reduced mitochondrial membrane potential ($\Delta\Psi_m$), were assessed using DiOC₆(3) (10 nM).³⁹ Apoptosis was also assessed using Hoechst 33342⁴⁰ with minor modifications. B-CLL cells (5 \times 10⁵) were suspended in

1 ml of medium, and incubated at 37°C for 1 min with Hoechst 33342 (1.5 μ g/ml), cooled on ice and re-suspended in cold PBS (700 μ l). Propidium iodide was added (5 μ g/ml), and the samples were analysed by flow cytometry. This method measures the increased cellular permeability of apoptotic cells⁴¹ and corresponds very well with increases in Annexin V binding.³⁹

Electron microscopy

Samples were prepared as described,⁴² with modifications. B-CLL cells (6 \times 10⁶) were spun down at 400 g in a centrifuge with a swing-out rotor. The pellets were fixed overnight at 4°C, with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. The samples were postfixed with 1% osmium tetroxide/1% potassium ferrocyanide, en bloc stained with 5% uranyl acetate, and embedded in Taab epoxy resin (Taab Laboratories, Aldermaston, UK).

Western blot analysis

Cell samples were prepared and proteins resolved on 10–15% SDS-polyacrylamide gels and blotted on to nitrocellulose membranes (Hybond C-extra; Amersham, Bucks, UK).⁴³ Caspases were detected as previously described.¹³ The rabbit polyclonal antibody to caspase-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), recognises the intact proform and the p14 small subunit.⁴³ Apaf-1 was detected using a mouse monoclonal antibody to human Apaf-1 (R&D Systems, Minneapolis, MN, USA). Western blots were developed using enhanced chemiluminescence (ECL; Amersham) according to the manufacturer's instructions.

Fluorimetric assay of caspase activity

DEVDase activity (ie primarily caspases-3 and -7) of lysates or column fractions was measured ($\lambda_{ex}/\lambda_{em}$ = 405/510 nm) at 37°C in 96-well plates in 200 μ l of assay buffer (20 μ M Z-DEVD.AFC, 0.1% CHAPS, 10 mM DTT, 100 mM Hepes and 10% sucrose, pH 7.0) as described previously.¹⁹

Preparation of B-CLL lysates for in vitro activation studies

B-CLL cells were purified to the stage before negative selection as described above. These cells were >89% CD19⁺ and CD5⁺. Cell lysates (10 mg/ml protein) were then prepared as described previously⁴³ and activated by addition of dATP (2 mM), cytochrome c (0.25 mg/ml) and MgCl₂ (1 mM).

Chromatographic methods

Lysates were fractionated by size-exclusion chromatography, using an FPLC protein purification system on a Superose 6 High Resolution 10/30 column (Amersham Pharmacia Biotech, Herts, UK) as described previously.¹⁹ The Superose 6 column was eluted at 4°C, 0.4 ml/min, with 5% (w/v) sucrose, 0.1% (w/v) CHAPS, 20 mM Hepes/NaOH, 5 mM DTT, 50 mM NaCl, pH 7.0 and 0.5 ml fractions were collected. The

columns were calibrated with protein standards (Amersham Pharmacia Biotech) as described.¹⁹

Assay of apoptosome effector caspase-activating activity

This assay measures the ability of an assembled Apaf-1 complex to both process and activate effector caspases-3 and -7. In preliminary experiments, fractionated active apoptosome complexes required neither dATP/ATP nor cytochrome c in order to process added procaspases, presumably because these cofactors are only required for the correct oligomerization of Apaf-1. Column fractions (100 μ l aliquots), from apoptosome complexes isolated from apoptotic or dATP-activated lysates by Superose-6 gel filtration as described above, were incubated with procaspases (25 μ g protein) for 30 min before assaying for DEVDase activity. The partially purified procaspases-3, -7 and -9 were obtained as described previously.¹⁹

Preparation of cytosolic extracts for cytochrome c analysis

Cell cytosols for assessment of cytochrome c release were prepared as previously described.⁴⁴ Briefly, cell pellets were resuspended in 2 \times volume lysate buffer supplemented with 250 mM sucrose, and incubated for 30 min at 4°C and then homogenised with a Dounce homogeniser (40 strokes). The cell extract was then centrifuged at 14 000 g for 15 min and the supernatant removed and stored at -80°C until assayed for cytochrome c by Western blotting. Cytochrome c was detected using a mouse monoclonal antibody (Pharmingen, San Diego, CA, USA).

Results

Spontaneous apoptosis in purified B-CLL cells

Purified B-CLL cells from patients (Table 1) were cultured either alone, with prednisolone or chlorambucil, or with proteasome inhibitors, and apoptosis assessed by externalisation of phosphatidylserine.^{23,38} B-CLL cells apparently fail to die by apoptosis *in vivo*, yet culture of purified cells for 18 h resulted in a wide range of spontaneous apoptosis (4.7–

73.7%) (Table 1). High levels of spontaneous apoptosis have been observed in other studies with purified leukaemic B cells, compared with lower levels found using unfractionated mononuclear cells,⁴⁵ indicating that survival factors are present *in vivo* providing B-CLL tumour cells with a survival advantage.

Proteasome inhibitors are potent inducers of apoptosis in B-CLL cells

Neither chlorambucil nor prednisolone consistently induced apoptosis in purified B-CLL cells over 18 h, although in patient No. 7 (Table 1) prednisolone induced marked apoptosis. Interestingly, this patient's B-CLL cells were resistant to chlorambucil *in vivo* but underwent a marked lymphocytolysis when treated with high doses of prednisolone. This suggested that *in vitro* sensitivity might be an indicator of *in vivo* sensitivity to chemotherapeutic agents in B-CLL patients. In contrast, lactacystin induced extensive apoptosis in cells from all patients studied (Table 1 and data not shown). Lactacystin is a specific, irreversible inhibitor of the proteasome, binding covalently to the amino-terminal threonine of the mammalian proteasome subunit X and not inhibiting the *in vitro* activity of any other known protease.⁴⁶ Lactacystin induced a time- and concentration-dependent induction of apoptosis in B-CLL cells, with almost complete apoptosis being observed after 24 h at higher concentrations (5–10 μ M) (Figure 1a). B-CLL cells from all patients ($n=15$) tested in this study have demonstrated a similar sensitivity to lactacystin. The cell permeable reversible proteasome inhibitors, MG132 and LLnL,⁴⁷ also induced apoptosis (Table 1 and Figure 1b). MG132 caused a time- and concentration-dependent induction of apoptosis and was ~10-fold more potent than either LLnL or lactacystin. MG132 potentially induced apoptosis in B-CLL cells purified from all patients ($n=10$) tested in this study. Thus proteasome inhibitors induce apoptosis in B-CLL cells irrespective of the stage of the disease, or resistance to conventional chemotherapeutic agents.

Proteasome inhibitors induce an apoptotic morphology concomitant with loss in mitochondrial membrane potential and phosphatidylserine externalisation

Lactacystin and MG132 induced a time-dependent increase in the percentage of cells with decreased $\Delta\Psi_m$, high Hoechst

Table 1 Clinical information and summary of *in vitro* apoptosis sensitivity

Patient No.	Binet stage	WBC $\times 10^9/l$	% Apoptosis					
			Spon	Pd	Chl	Lacta	LLnL	MG132
1	C	120.0	6.5	12.8	5.2	89.2	97.5	—
2	A	23.7	34.1	26.8	24.9	99.5	98.8	—
3	A	40.9	47.0	42.9	46.0	99.2	97.3	—
4	A	26.2	53.8	68.8	65.2	97.7	97.7	—
5	A	20.5	73.7	69.2	84.3	98.0	98.8	—
6	A	39.0	12.3	18.2	16.1	91.4	92.6	91.9
7	C	21.4	28.1	76.8	27.7	88.6	96.1	—
8	B	78.0	8.8	—	—	92.5	96.3	94.9
9	A	22.2	4.7	—	—	—	—	86.7

Purified B-CLL cells were cultured for 18 h either alone (Spon) in the presence of prednisolone (20 μ M) (Pd), chlorambucil (10 μ M) (Chl), lactacystin (10 μ M) (Lacta), LLnL (10 μ M) or MG132 (1 μ M). Apoptosis was assessed by externalisation of phosphatidylserine.

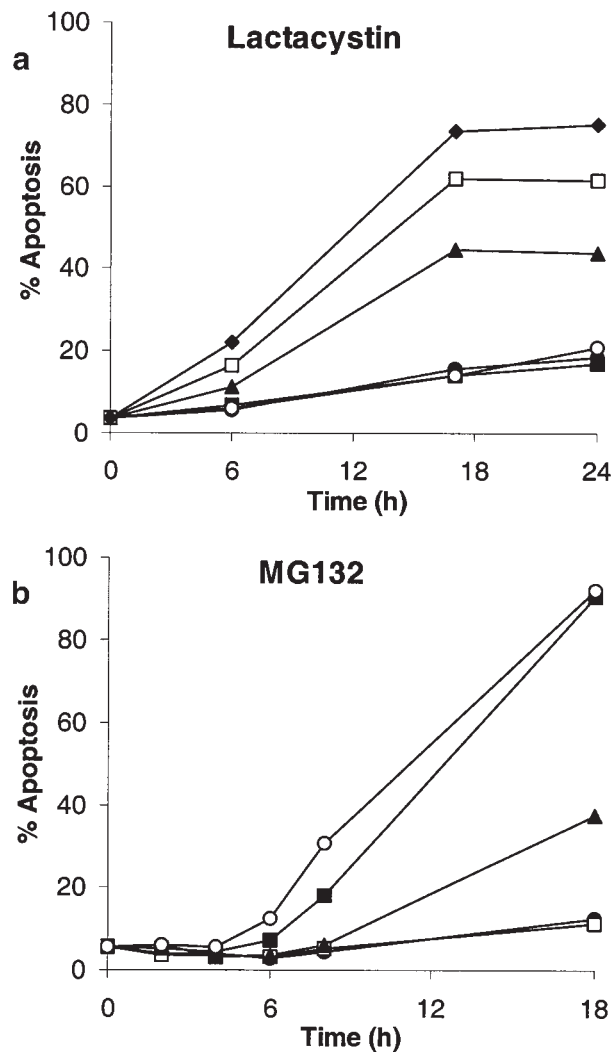


Figure 1 Proteasome inhibitors cause a time- and concentration-dependent induction of apoptosis in B-CLL cells. Freshly isolated purified B-CLL cells were incubated (a) from 0 to 24 h either alone (●) or in the presence of lactacystin, 0.625 μM (■), 1.25 μM (○), 2.5 μM (▲), 5 μM (□) or 10 μM (◆). (b) Similarly, B-CLL cells from another patient were incubated from 0 to 18 h either alone (●) or in the presence of MG132, 0.125 μM (□), 0.25 μM (▲), 0.5 μM (■) or 1.0 μM (○). Apoptotic cells were assessed by increased externalisation of phosphatidylserine as described in Materials and methods.

33342 staining and phosphatidylserine exposure (Figure 2). A reduction in $\Delta\Psi_m$ is an early event in the induction of apoptosis in many different systems.⁴⁸ Increased uptake of Hoechst 33342 has also been used to measure apoptosis in some systems.^{40,41} Thus proteasome inhibitor-induced apoptosis in B-CLL cells could be determined by three independent quantitative methods. Finally, both lactacystin and MG132 induced typical apoptotic ultrastructure in B-CLL cells (Figure 3). In comparison to control cells (Figure 3a), cells treated with MG132 (Figure 3b) and lactacystin (Figure 3c) exhibited chromatin condensation, decrease in cell volume and cytoplasmic vacuolation, resulting from distension of some cisternae of the endoplasmic reticulum. Mitochondria appeared normal and intact, except in cells showing the onset of secondary necrosis. Lactacystin (10 μM) inhibited proteasomal activity of B-CLL cells as early as 30 min after exposure (data not shown) and clearly prior to the onset of apoptosis.

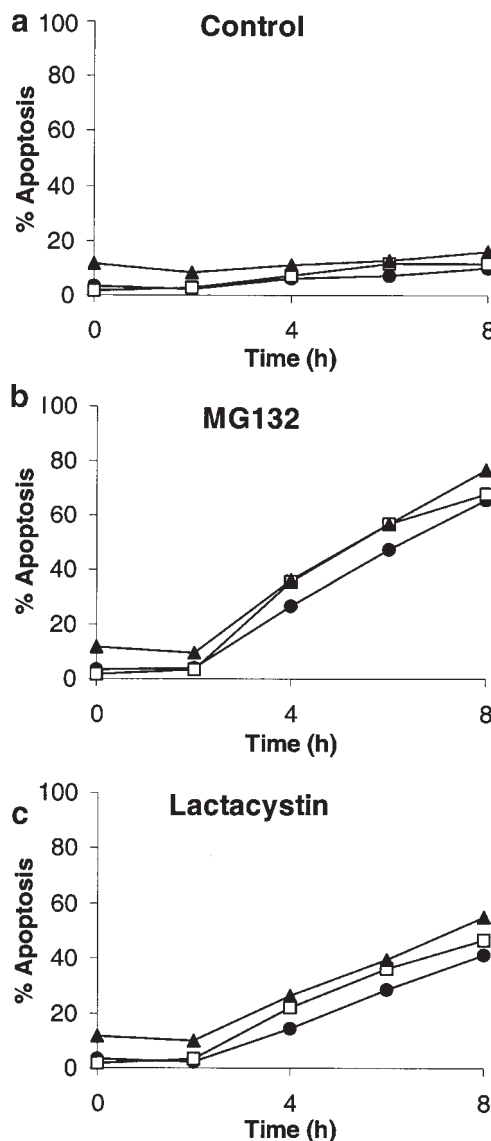


Figure 2 Proteasome inhibitors induce apoptosis in B-CLL cells as assessed by externalisation of phosphatidylserine, loss of mitochondrial membrane potential ($\Delta\Psi_m$), and staining with Hoechst 33342. Freshly isolated purified B-CLL cells were incubated for 0–8 h either alone (a), with MG132 (1 μM) (b), and with lactacystin (10 μM) (c). At the indicated times, apoptosis was quantified by externalisation of phosphatidylserine (●), by loss of $\Delta\Psi_m$ (▲) assessed by decrease of binding of DiOC₆(3), and by staining with Hoechst 33342 (□) as described in Materials and methods. All these independent measures of apoptosis gave very similar results. The data are representative of three experiments using cells from different patients.

MG132 induces caspase-independent cytochrome c release from mitochondria

MG132 induced a release of mitochondrial cytochrome c compared with control cells (Figure 4, compare lanes 2 and 3), which was not prevented by the poly-caspase inhibitor Z-VAD.fmk (200 μM)⁴⁹ (Figure 4, lane 4). Z-VAD.fmk clearly entered the cell and inhibited caspase activity as it inhibited many other features of the apoptotic phenotype (Figures 5 and 6). Cytochrome oxidase, an inner mitochondrial membrane protein, was detected in the cell pellets containing the mito-

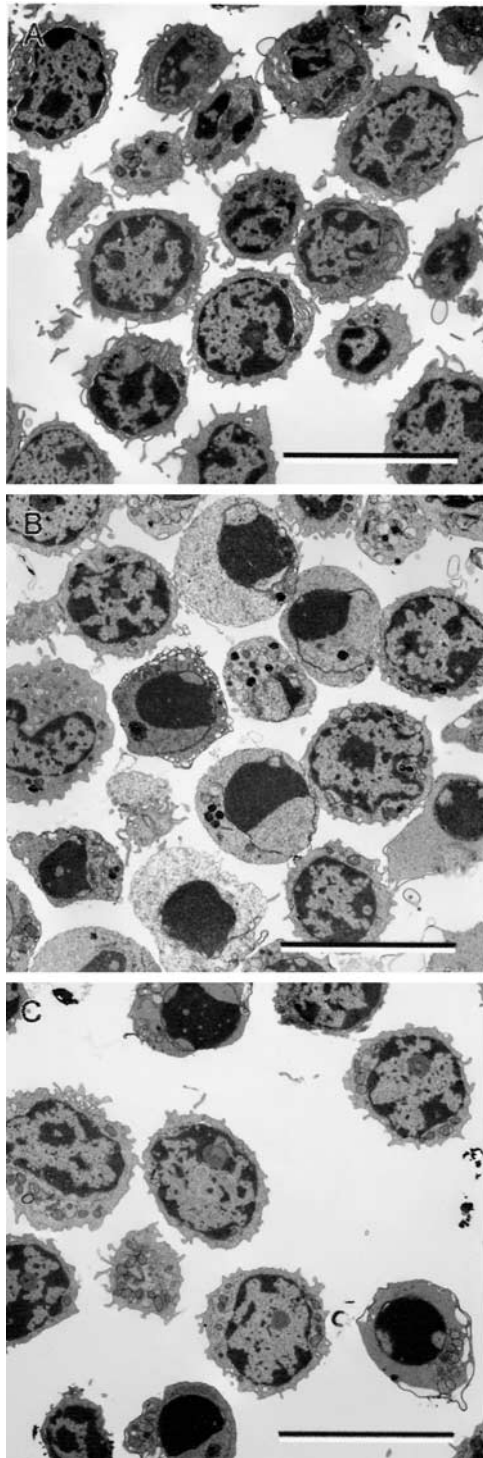


Figure 3 Proteasome inhibitors induce apoptotic morphology in B-CLL cells. Freshly isolated purified B-CLL cells (a) were exposed to (b) MG132 (1 μM) or (c) lactacystin (10 μM) for 8 h. Many of the treated cells displayed characteristic apoptotic nuclear morphology. Bars: 10 μm .

chondria but not in the cytosolic extracts (data not shown) confirming that the mitochondria remained intact during the preparation of the extracts. The data suggested that the induction of apoptosis by proteasome inhibitors was preceded by a caspase-independent release of mitochondrial cytochrome c. The data agree with a previous study demonstrating that

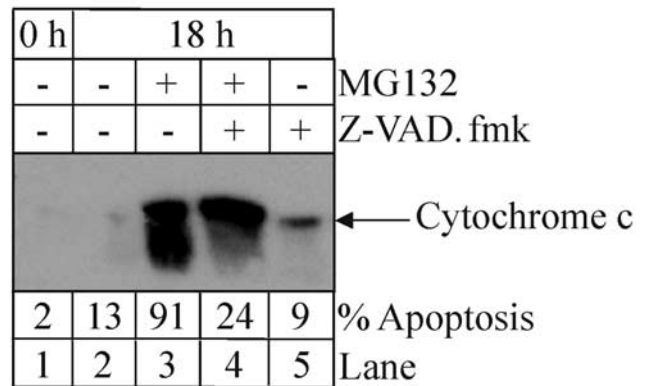


Figure 4 Proteasome inhibitor induced apoptosis is accompanied by release of mitochondrial cytochrome c. B-CLL cells were incubated either alone or with MG132 (1 μM) or Z-VAD.fmk (200 μM) as indicated. Apoptosis was assessed by increased externalisation of phosphatidylserine. Cell extracts were prepared and the cell pellets and cytosolic extracts examined by immunoblotting for the presence of cytochrome c and cytochrome oxidase (data not shown). The results are typical of three experiments using cells isolated from three different B-CLL patients.

proteasomal inhibition resulted in mitochondrial cytochrome c release.⁴

Proteasome inhibitor-induced apoptosis is caspase-dependent in B-CLL cells

Although pre-treatment with Z-VAD.fmk did not inhibit mitochondrial cytochrome c release, it completely abrogated MG132-induced apoptosis up to 8 h and largely inhibited it at 18 h (Figure 5a). Treatment with MG132 caused a time-dependent increase in DEVDase, a measure of the activity of effector caspases -3 and -7, which was completely abolished by Z-VAD.fmk (Figure 5b). These data supported the involvement of effector caspases in the execution phase of MG132-induced apoptosis.

Activation of a caspase cascade in proteasome inhibitor treated B-CLL cells

B-CLL cells were examined for the processing of various initiator and effector caspases during the induction of apoptosis. Untreated cells contained the 46 kDa proform of caspase-9 (Figure 6a lane 1), which on exposure to MG132 was processed to both an ~35 kDa fragment and smaller amounts of an ~37 kDa fragment (Figure 6a lanes 3–6), resulting from cleavage between the large and small subunits at Asp 315 and Asp 330, respectively.⁵⁰ A time-dependent processing of caspase-9 was observed which was evident at 4 h, and almost complete at 18 h (Figure 6a lanes 2–6). Treatment with Z-VAD.fmk did not completely inhibit the MG132 induced processing of caspase-9, although at 18 h some inhibition was evident as judged by more zymogen being present (Figure 6a lanes 7–11). As caspase-9 processing was not completely inhibited by Z-VAD.fmk, we cannot totally exclude the possibility that mitochondrial cytochrome c release was caused by active caspase-9. However, this is unlikely as several studies have demonstrated that cytochrome c release pre-

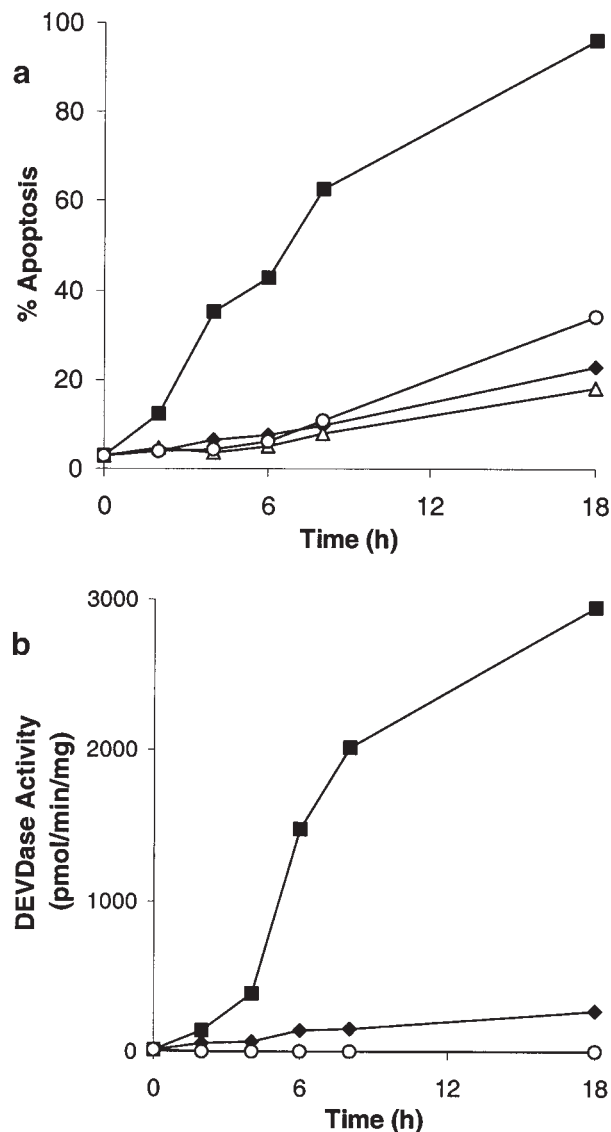


Figure 5 Proteasome inhibitor-induced apoptosis is caspase dependent. Freshly isolated purified B-CLL cells were incubated for up to 18 h either alone (◆) or in the presence of MG132 (1 μ M) (■), MG132 (1 μ M) and Z-VAD.fmk (200 μ M) (○), or with Z-VAD.fmk (200 μ M) alone (△). Apoptotic cells were assessed by increased externalisation of phosphatidylserine (a). Extracts from these cells were assayed for DEVDase activity as described in Materials and methods (b). The data are representative of three experiments using cells from different patients.

cedes activation of caspase-9 in a post mitochondrial caspase-activation pathway.^{11,13}

Caspase-3 is normally processed at Asp-175 between the large and small subunits yielding a p20 subunit, which is further processed at Asp-9 and Asp-28 to yield p19 and p17 large subunits.⁵¹ Pro-caspase-3 was present in control B-CLL cells primarily as its inactive zymogen (Figure 6b lane 1), which on exposure to MG132 was cleaved to form two fragments of ~19 kDa (p19) and ~17 kDa (p17) (Figure 6b). Some processing was observed at 4 h and was complete at 18 h (Figure 6b lanes 3–6). In the presence of Z-VAD.fmk, MG132 induced a time-dependent processing of pro-caspase-3 to the ~20 kDa (p20) fragment (Figure 6b lanes 7–11), indicative of cleavage of pro-caspase-3 between the large and small sub-

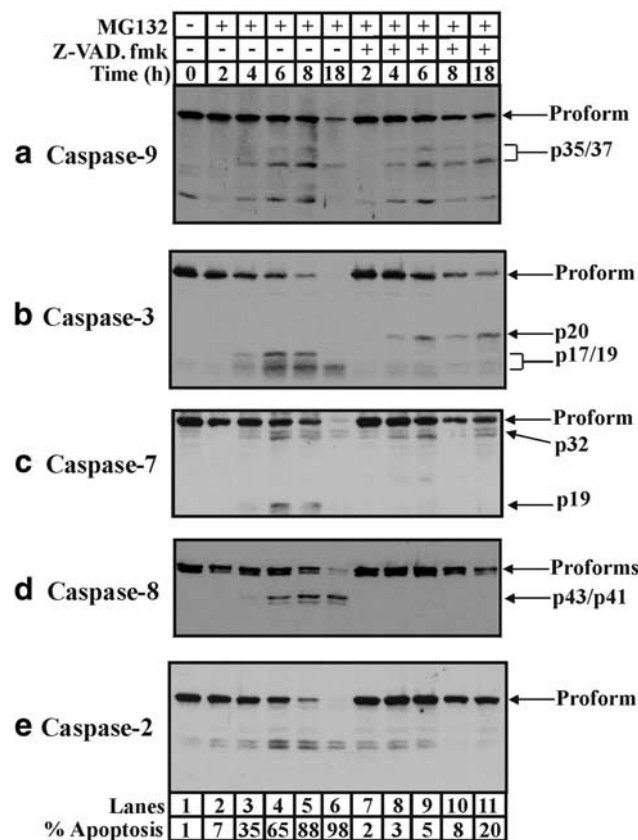


Figure 6 MG132 induces a time-dependent processing of caspases. B-CLL cells were incubated for up to 18 h either alone or in the presence of MG132 (1 μ M), with or without Z-VAD.fmk (200 μ M) as indicated. Cells were then analysed by immunoblotting for the processing of (a) caspase-9, (b) caspase-3, (c) caspase-7, (d) caspase-8 and (e) caspase-2 as described in Materials and methods. The proforms and processed forms of the caspases are indicated. A shorter exposure of the film showed that the proform of caspase-8 comprised two bands of ~55 and 53 kDa. The data are representative of three experiments using cells from different patients. Apoptosis was assessed by externalisation of phosphatidylserine.

units by caspase-9,⁵⁰ which was not totally abrogated by the caspase inhibitor (Figure 6a lanes 7–11). However, the accumulation of the p20 fragment, without further processing to the p19 and p17 fragments, demonstrated that Z-VAD.fmk blocked the autocatalytic activity of caspase-3.^{51,52} Z-VAD.fmk also abolished MG132-induced DEVDase activity in B-CLL cells (Figure 5b) demonstrating that the p20 fragment of caspase-3 was not catalytically active. Following exposure to MG132, a time-dependent loss of procaspase-7 was observed together with formation of an ~19 kDa (p19) fragment (Figure 6c), which corresponds to the catalytically active large subunit.⁵¹ Z-VAD.fmk blocked the processing of pro-caspase-7 to the p19 fragment (Figure 6c lanes 7–11).

Caspase-8 was present in untreated B-CLL cells primarily as two isoforms of ~55 and 53 kDa (Figure 6d lane 1), most probably corresponding to caspase-8a and -8b.⁵³ Following exposure to MG132, caspase-8 was also processed in a time-dependent manner (Figure 6d) to two fragments of ~43 and 41 kDa (p43 and p41, respectively), corresponding to cleavage of both caspase-8a and -8b between the large and small subunits. Caspase-8 was first processed at 4 h, and almost completely processed after 18 h (Figure 6d lanes 3–6). Z-

VAD.fmk completely inhibited the time-dependent processing of caspase-8 induced by MG132 (Figure 6d lanes 7–11).

Caspase-2 was present in B-CLL cells primarily as a 48 kDa proform (Figure 6e lane 1), which on exposure to MG132, showed a time-dependent loss from 4 to 18 h (Figure 6e lanes 3–6), which was largely inhibited by Z-VAD.fmk (Figure 6e lanes 7–11). The cells also contained an immunologically reactive protein of ~33 kDa (Figure 6e), which we observed in an earlier study.²³ From the data it is not clear whether this represents a processed form of caspase-2. The processing of caspases-9, -3, -7, -8 and -2 in lactacystin-treated B-CLL cells had the same profile as that observed in MG132-treated cells (data not shown).

The data clearly demonstrated that the treatment of B-CLL cells with MG132 resulted in the initiation of a caspase cascade in which the processing of caspase -9 was independent of caspases -8, -2, -3 and -7. This strongly indicated that proteasome inhibitors induce apoptosis in B-CLL cells via a caspase cascade with caspase-9 as the initiator. Recently, we have shown that some chemical stimuli induce apoptosis by post-mitochondrial formation of a caspase-activating apoptosome complex.^{16,19} Therefore, we investigated whether a similar mechanism operated in B-CLL cells.

Formation of a functional apoptosome in B-CLL cell lysates

First, we used dATP-activated B-CLL lysates to determine whether they were capable of assembling a functional apoptosome. In control lysates, both procaspases -9 and -3 eluted as their free unprocessed zymogens and Apaf-1 eluted as a monomer (Figure 7a). Following dATP activation, a marked change in the elution behaviour of Apaf-1 was observed, with it now being oligomerised and completely associated with ~1.4 MDa and ~700 kDa apoptosome complexes (Figure 7b, fractions 6–9 and fractions 10–16, respectively). After dATP activation, procaspase-9 was partially processed to its p35/p37 subunits, which eluted as free processed caspases (Figure 7b), with very little of the processed fragments associated with the apoptosome complexes. Procaspase-3 was completely processed to its catalytically active large subunits (p17/19) (Figure 7b), which co-eluted with all the DEVDase activity (Figure 7c, fractions 22–28, $M_r = \sim 60000$). Most of the biological activity as assessed by ability to process effector caspases was associated with the ~700 kDa Apaf-1 containing apoptosome complex although a small amount of activity was also associated with the ~1.4 MDa apoptosome complex (Figure 7c). Thus following dATP activation, lysates from B-CLL cells formed a fully functional, caspase activating ~700 kDa Apaf-1 containing apoptosome complex.

An ~700 kDa apoptosome complex is formed in apoptotic B-CLL cells

In order to determine whether a functional apoptosome was formed in apoptotic cells, B-CLL cells were treated with MG132. In control cells, Apaf-1 eluted as a monomer and procaspases-9 and -3 eluted as free caspases (Figure 8a) as previously observed in lysates (Figure 7a). Following exposure to MG132 (10 μM) for 5 h, which resulted in ~40% apoptosis, processing of caspases-9 and -3 to their large subunits was observed (Figure 8b). The processed caspase-3 corresponded to the DEVDase activity (compare Figure 8b and c, fractions

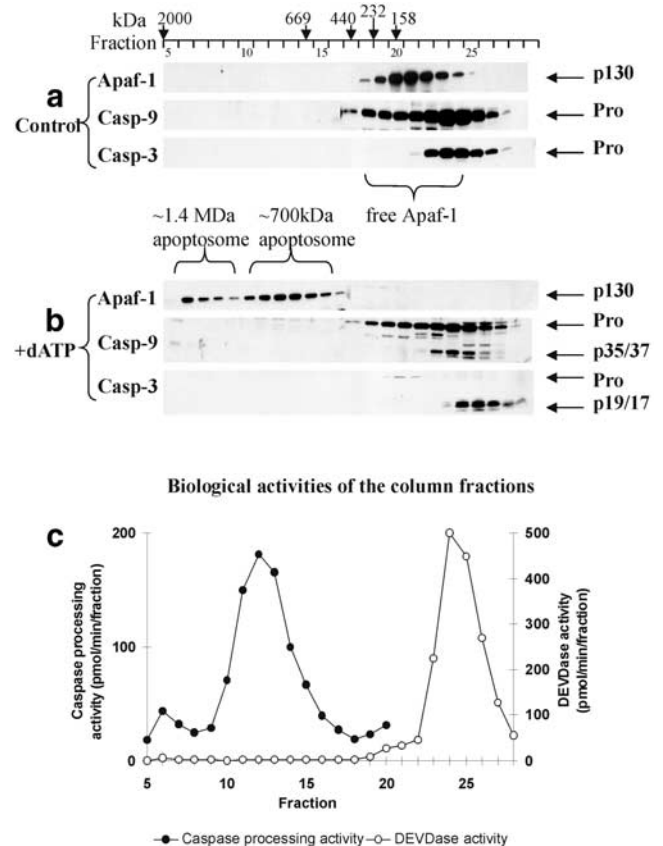


Figure 7 An ~700 kDa apoptosome complex is formed following dATP activation of B-CLL cell lysates. Lysates (10 mg/ml protein) from B-CLL cells were incubated with or without dATP (2 mM), cytochrome c (0.25 mg/ml) and MgCl_2 (1 mM) for 1 h and the lysate (2.9 mg) separated on a Superose 6 column and immunoblotted. (a) Apaf-1 from control lysates eluted as a monomer and caspases-3 and -9 as their unprocessed zymogens. (b) After dATP activation, Apaf-1 eluted in two apoptosome complexes. Caspase-9 was partially processed to its p35/p37 subunits, which eluted as free heterotetramers. Caspase-3 was entirely processed to its active large subunit. (c) The ~700 kDa but not the ~1.4 MDa apoptosome possessed most of the ability to process and activate effector caspases. The Apaf-1-containing fractions (5–20, b) were assayed for caspase processing activity by measuring DEVDase activity (●●) produced when the fractions were incubated with 'free caspases' as described in the Methods. Thus, the ~700 kDa apoptosome complex (fractions 10–16) initially had very little or no DEVDase activity but possessed the inherent ability to activate exogenous effector caspases. All the fractions from (b) were also initially assayed for DEVDase activity (○○). The NaCl in the elution buffer resulted in the DEVDase (ie free active effector caspases-3 and -7) activity eluting in fractions 23–28. The positions of M_r markers are indicated with arrows. The data are representative of three experiments using cell lysates from different patients.

22–26). Some Apaf-1 clearly oligomerised to an ~700 kDa complex (Figure 8b, fractions 10–16), whilst most still eluted as free monomeric Apaf-1. The ~700 kDa Apaf-1 containing apoptosome complex possessed caspase processing activity (Figure 8c).

With some patients it was more difficult to detect the formation of the apoptosome in response to treatment with MG132. The formation of a biologically active apoptosome was evident in activated lysates (Figure 7) produced from cells isolated from patient No. 27. Cells from this patient were also induced to undergo ~40% apoptosis by treatment with MG132 (1 μM) for 10 h, which resulted in processing of casp-

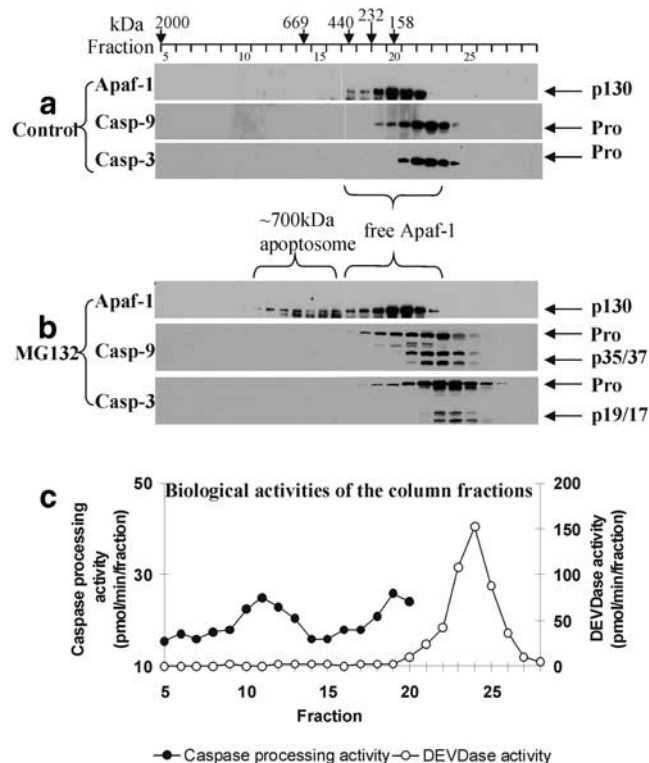


Figure 8 An ~700 kDa apoptosome complex is formed in apoptotic B-CLL cells. B-CLL cells were incubated for 5 h either (a), or (b) with MG132 (10 μ M) to induce ~40% apoptosis. Lysates were then prepared and fractionated by Superose 6 gel filtration. The fractions were run on SDS-PAGE and immunoblotted for Apaf-1, caspases-9 and -3, and assayed for effector caspase (DEVDase) and caspase processing activity. In control cells (a), Apaf-1 eluted as the monomeric form and there was no processing of caspases-9 and -3. In MG132-treated cells (b), Apaf-1 eluted both as its monomeric form and as an ~700 kDa apoptosome complex, and caspases-9 and -3 were processed/activated. Caspase processing activity was primarily associated with the ~700 kDa apoptosome complex (c). Control cell lysates did not have any detectable caspase processing activity (data not shown).

ases-3 and -9 to their p17/p19 and p35/p37 large subunits, respectively, which eluted as free caspases (Figure 9a, fractions 19–28). In this patient (No. 27), only a very small amount of oligomerised Apaf-1 eluted at ~700 kDa (Figure 9a, fractions 10–16) with a correspondingly small amount of caspase processing activity (Figure 9b, fractions 10–16). Interestingly, the Apaf-1 antibody also detected an immunoreactive fragment of ~30 kDa (Figure 9a, fractions 25–29), which was not present in the control cells (Figure 7a) and corresponded to an ~30 kDa cleavage fragment of Apaf-1.⁵⁴ The data indicated that variable amounts of an Apaf-1 caspase activating apoptosome complex are formed in B-CLL cells induced to undergo apoptosis with proteasome inhibitors.

Discussion

Proteasome inhibitors potently induce apoptosis in B-CLL cells assessed by a number of criteria (Figures 1–5). Although proteasome inhibitors have been reported to inhibit apoptosis in quiescent or differentiated cells,^{32,33} this clearly cannot be a general phenomenon as B-CLL is a disease characterised by quiescent malignant B lymphocytes largely arrested in the

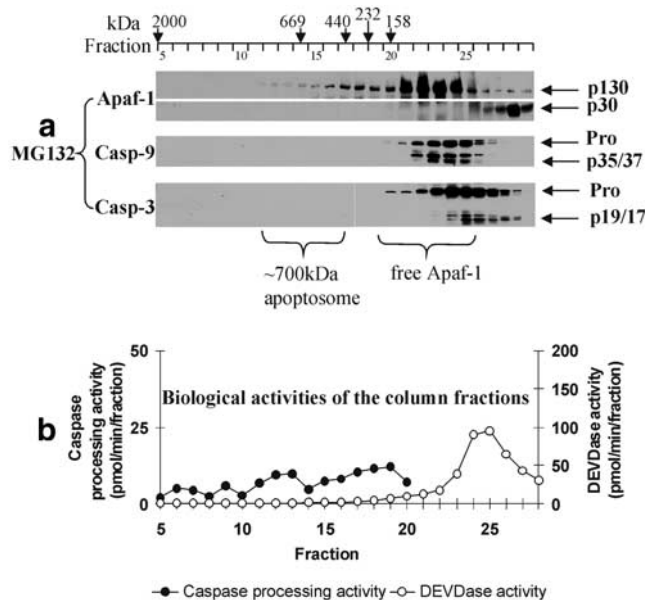


Figure 9 Minimal formation of an ~700 kDa apoptosome complex in some apoptotic B-CLL cells. B-CLL cells were incubated with MG132 (1 μ M) for 10 h to induce ~40% apoptosis. Lysates were prepared and fractions assayed as described in the legend to Figure 8. In control cells from this patient (Figure 7a), Apaf-1 eluted as a monomer and there was no processing of caspases-9 and -3. In MG132-treated cells (a), Apaf-1 eluted primarily as a monomer together with very small amounts of an ~700 kDa apoptosome complex. A cleaved ~30 kDa Apaf-1 fragment was also observed. Caspases-9 and -3 were processed to the catalytically active large subunits. There was very little caspase processing activity associated with the ~700 kDa apoptosome complex (b). Control cell lysates did not have any detectable caspase processing activity (Figure 7).

G0/G1 phase of the cell cycle.^{1–3} We describe for the first time the presence of five unprocessed caspases in control B-CLL cells all of which were processed/activated on induction of apoptosis. Our data support the hypothesis of a caspase cascade with caspase-9 as the initiator caspase. Some support for this hypothesis was provided by the observation that caspase-9 was still partially processed in the presence of Z-VAD.fmk (Figure 6). Processed caspase-9 activates both caspases-3 and -7 following cleavage between their large and small subunits. These effector caspases then activate directly or indirectly caspases-8 and -2. This most probably occurs indirectly by caspase-6, as in dATP-activated lysates caspase-3 activates caspase-6, which in turn activates caspases-2 and -8.⁵⁵ Taken together, our data suggests that proteasome inhibitor-induced apoptosis of B-CLL cells proceeds via perturbation of mitochondria, resulting in cytochrome c release and initiation of a caspase cascade with caspase-9 as the initiator caspase.

Using both dATP-activated lysates and apoptotic B-CLL cells, we have demonstrated the formation of an ~700 kDa Apaf-1 containing caspase-activating complex (Figures 7–9) similar to that previously demonstrated in human monocytic THP.1 cells and in rat hepatoma cells.^{19,56} After dATP activation, we also observed formation of a larger ~1.4 MDa Apaf-1 apoptosome complex with reduced caspase processing activity. The lower activity of the larger complex appears to be due to inappropriate oligomerization of Apaf-1.⁵⁴ Interestingly, little if any processed or unprocessed caspase-9 was observed in either of the apoptosome complexes in lysates or cells (Figures 7–9). Caspase-9 binds tightly to Apaf-1 through

CARD-CARD interactions between the N-termini of caspase-9 and Apaf-1,^{57,58} suggesting that additional factors may be present in B-CLL cells that weaken the CARD-CARD interaction between caspase-9 and Apaf-1, resulting in dissociation of caspase-9 from apoptosome complexes during purification. Caspase-9 activity is dependent on it being bound to Apaf-1.^{59,60} However, we have recently shown that processed caspase-9 can be recruited to the apoptosome complex and subsequently activate caspase-3.⁶¹ Thus, the free processed caspase-9 present in cells and lysates from B-CLL cells could be similarly recruited to the apoptosome.

In B-CLL cells induced to undergo apoptosis by exposure to proteasome inhibitors, varying amounts of the biologically active ~700 kDa Apaf-1 containing complex were observed and the majority of the cellular Apaf-1 remained as its monomeric form (Figures 8 and 9), most probably because only 40% of the cells were apoptotic at the time-point examined. In addition, amplification loops in caspase cascades may enable a modest level of apoptosome formation to initiate extensive caspase activation. Nevertheless, in cells from some patients induced to undergo similar levels of apoptosis, it was more difficult to detect formation of an active apoptosome (Figure 9). In these experiments Apaf-1 was cleaved by caspase-3 to an ~30 kDa Apaf-1 immunoreactive fragment.⁵⁴ Although we had previously shown this fragment to associate with the ~1.4 MDa apoptosome complex, it was possible that the apoptosome may be rapidly degraded by active caspases in B-CLL cells making it difficult to detect. Alternatively, we cannot totally exclude the possibility that in cells from some patients, proteasome inhibitors activate caspases by an alternative mechanism not involving apoptosome formation.

In conclusion, our data indicated that proteasome inhibitors induce apoptosis in B-CLL cells via disruption of the mitochondria and release of cytochrome c, resulting in the formation of an Apaf-1 containing ~700 kDa caspase-activating complex that activates the initiator caspase-9. In B-CLL cells from some patients this large caspase-activating complex may be rapidly degraded by active caspases.

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References

- Rozman C, Montserrat E. Chronic lymphocytic leukemia. *N Engl J Med* 1995; **333**: 1052-1057.
- Dameshek W. Chronic lymphocytic leukemia – an accumulative disease of immunologically incompetent lymphocytes. *Blood* 1967; **29**: 566-584.
- Reed JC. Molecular biology of chronic lymphocytic leukemia. *Semin Oncol* 1998; **25**: 11-18.
- Chandra J, Niemer I, Gilbreath J, Kliche KO, Andreeff M, Freireich EJ, Keating M, McConkey DJ. Proteasome inhibitors induce apoptosis in glucocorticoid-resistant chronic lymphocytic leukemic lymphocytes. *Blood* 1998; **92**: 4220-4229.
- Delic J, Masdehors P, Omura S, Cosset JM, Dumont J, Binet JL, Magdelenat H. The proteasome inhibitor lactacystin induces apoptosis and sensitizes chemo- and radioresistant human chronic lymphocytic leukaemia lymphocytes to TNF-alpha-initiated apoptosis. *Br J Cancer* 1998; **77**: 1103-1107.
- Masdehors P, Omura S, Merle-Beral H, Mentz F, Cosset JM, Dumont J, Magdelenat H, Delic J. Increased sensitivity of CLL-derived lymphocytes to apoptotic death activation by the proteasome-specific inhibitor lactacystin. *Br J Haematol* 1999; **105**: 752-757.
- Cohen GM. Caspases: the executioners of apoptosis. *Biochem J* 1997; **326**: 1-16.
- Nicholson DW. Caspase structure, proteolytic substrates, and function during apoptotic cell death. *Cell Death Differ* 1999; **6**: 1028-1042.
- Boldin MP, Goncharov TM, Goltsev YV, Wallach D. Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. *Cell* 1996; **85**: 803-815.
- Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko A, Ni J, Scaffidi C, Bretz JD, Zhang M, Gentz R, Mann M, Krammer PH, Peter ME, Dixit VM. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. *Cell* 1996; **85**: 817-827.
- Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 1997; **91**: 479-489.
- Zou H, Henzel WJ, Liu X, Lutschg A, Wang X. Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell* 1997; **90**: 405-413.
- Sun XM, MacFarlane M, Zhuang J, Wolf BB, Green DR, Cohen GM. Distinct caspase cascades are initiated in receptor-mediated and chemical-induced apoptosis. *J Biol Chem* 1999; **274**: 5053-5060.
- Green DR, Reed JC. Mitochondria and apoptosis. *Science* 1998; **281**: 1309-1312.
- Bratton SB, MacFarlane M, Cain K, Cohen GM. Protein complexes activate distinct caspase cascades in death receptor and stress-induced apoptosis. *Exp Cell Res* 2000; **256**: 27-33.
- Cain K, Brown DG, Langlais C, Cohen GM. Caspase activation involves the formation of the aposome, a large (~700 kDa) caspase-activating complex. *J Biol Chem* 1999; **274**: 22686-22692.
- Saleh A, Srinivasula SM, Acharya S, Fishel R, Alnemri ES. Cytochrome c and dATP-mediated oligomerization of Apaf-1 is a prerequisite for procaspase-9 activation. *J Biol Chem* 1999; **274**: 17941-17945.
- Zou H, Li Y, Liu X, Wang X. An APAF-1/cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. *J Biol Chem* 1999; **274**: 11549-11556.
- Cain K, Bratton SB, Langlais C, Walker G, Brown DG, Sun XM, Cohen GM. Apaf-1 oligomerizes into biologically active ~700 kDa and inactive ~1.4 MDa apoptosome complexes. *J Biol Chem* 2000; **275**: 6067-6070.
- Bellosillo B, Dalmau M, Colomer D, Gil J. Involvement of CED-3/ICE proteases in the apoptosis of B-chronic lymphocytic leukemia cells. *Blood* 1997; **89**: 3378-3384.
- Chandra J, Gilbreath J, Freireich EJ, Kliche KO, Andreeff M, Keating M, McConkey DJ. Protease activation is required for glucocorticoid-induced apoptosis in chronic lymphocytic leukemic lymphocytes. *Blood* 1997; **90**: 3673-3681.
- Kitada S, Andersen J, Akar S, Zapata JM, Takayama S, Krajewski S, Wang HG, Zhang X, Bullrich F, Croce CM, Rai K, Hines J, Reed JC. Expression of apoptosis-regulating proteins in chronic lymphocytic leukemia: correlations with *in vitro* and *in vivo* chemoresponses. *Blood* 1998; **91**: 3379-3389.
- King D, Pringle JH, Hutchinson M, Cohen GM. Processing/activation of caspases, -3 and -7 and -8 but not caspase-2, in the induction of apoptosis in B-chronic lymphocytic leukemia cells. *Leukemia* 1998; **12**: 1553-1560.
- Baumeister W, Walz J, Zuhl F, Seemuller E. The proteasome: paradigm of a self-compartmentalizing protease. *Cell* 1998; **92**: 367-380.
- Drexler HCA. Programmed cell death and the proteasome. *Apoptosis* 1998; **3**: 1-7.
- Orlowski RZ. The role of the ubiquitin-proteasome pathway in apoptosis. *Cell Death Differ* 1999; **6**: 303-313.
- Imajoh-Ohmi S, Kawaguchi T, Sugiyama S, Tanaka K, Omura S, Kikuchi H. Lactacystin, a specific inhibitor of the proteasome, induces apoptosis in human monoblast U937 cells. *Biochem Biophys Res Commun* 1995; **217**: 1070-1077.

- 28 Shinohara K, Tomioka M, Nakano H, Tone S, Ito H, Kawashima S. Apoptosis induction resulting from proteasome inhibition. *Biochem J* 1996; **317**: 385–388.
- 29 Drexler HC. Activation of the cell death program by inhibition of proteasome function. *Proc Natl Acad Sci USA* 1997; **94**: 855–860.
- 30 Lopes UG, Erhardt P, Yao R, Cooper GM. p53-dependent induction of apoptosis by proteasome inhibitors. *J Biol Chem* 1997; **272**: 12893–12896.
- 31 Tanimoto Y, Onishi Y, Hashimoto S, Kizaki H. Peptidyl aldehyde inhibitors of proteasome induce apoptosis rapidly in mouse lymphoma RVC cells. *J Biochem* 1997; **121**: 542–549.
- 32 Grimm LM, Goldberg AL, Poirier GG, Schwartz LM, Osborne BA. Proteasomes play an essential role in thymocyte apoptosis. *EMBO J* 1996; **15**: 3835–3844.
- 33 Sadoul R, Fernandez PA, Quiquerez AL, Martinou I, Maki M, Schroter M, Becherer JD, Imler M, Tschopp J, Martinou JC. Involvement of the proteasome in the programmed cell death of NGF-deprived sympathetic neurons. *EMBO J* 1996; **15**: 3845–3852.
- 34 Herrmann JL, Briones F, Brisbay S, Logothetis CJ, McDonnell TJ. Prostate carcinoma cell death resulting from inhibition of proteasome activity is independent of functional Bcl-2 and p53. *Oncogene* 1998; **17**: 2889–2899.
- 35 Orlowski RZ, Eswara JR, Lafond-Walker A, Grever MR, Orlowski M, Dang CV. Tumor growth inhibition induced in a murine model of human Burkitt's lymphoma by a proteasome inhibitor. *Cancer Res* 1998; **58**: 4342–4348.
- 36 Adams J, Palombella VJ, Sausville EA, Johnson J, Destree A, Lazarus DD, Maas J, Pien CS, Prakash S, Elliott PJ. Proteasome inhibitors: a novel class of potent and effective antitumor agents. *Cancer Res* 1999; **59**: 2615–2622.
- 37 Binet JL, Auquier A, Dighiero G, Chastang C, Piguat H, Goasguen J, Vaugier G, Potron G, Colona P, Oberling F, Thomas M, Tchernia G, Jacquillat C, Boivin P, Lesty C, Duault MT, Monconduit M, Belabbes S, Gremy F. A new prognostic classification of chronic lymphocytic leukemia derived from a multivariate survival analysis. *Cancer* 1981; **48**: 198–206.
- 38 Martin SJ, Reutelingsperger CP, McGahon AJ, Rader JA, van Schie RC, LaFace DM, Green DR. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J Exp Med* 1995; **182**: 1545–1556.
- 39 Zhuang J, Ren Y, Snowden RT, Zhu H, Gogvadze V, Savill JS, Cohen GM. Dissociation of phagocyte recognition of cells undergoing apoptosis from other features of the apoptotic program. *J Biol Chem* 1998; **273**: 15628–15632.
- 40 Sun XM, Snowden RT, Skilleter DN, Dinsdale D, Ormerod MG, Cohen GM. A flow-cytometric method for the separation and quantitation of normal and apoptotic thymocytes. *Anal Biochem* 1992; **204**: 351–356.
- 41 Ormerod MG, Sun XM, Snowden RT, Davies R, Fearnhead H, Cohen GM. Increased membrane permeability of apoptotic thymocytes: a flow cytometric study. *Cytometry* 1993; **14**: 595–602.
- 42 MacFarlane M, Merrison W, Dinsdale D, Cohen GM. Active caspases and cleaved cytokeratins are sequestered into cytoplasmic inclusions in TRAIL-induced apoptosis. *J Cell Biol* 2000; **148**: 1239–1254.
- 43 MacFarlane M, Cain K, Sun XM, Alnemri ES, Cohen GM. Processing/activation of at least four interleukin-1beta converting enzyme-like proteases occurs during the execution phase of apoptosis in human monocytic tumor cells. *J Cell Biol* 1997; **137**: 469–479.
- 44 Bossy-Wetzell E, Newmeyer DD, Green DR. Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. *EMBO J* 1998; **17**: 37–49.
- 45 Dancescu M, Rubio-Trujillo M, Biron G, Bron D, Delespesse G, Sarfati M. Interleukin 4 protects chronic lymphocytic leukemic B cells from death by apoptosis and upregulates Bcl-2 expression. *J Exp Med* 1992; **176**: 1319–1326.
- 46 Fenteany G, Schreiber SL. Lactacystin, proteasome function, and cell fate. *J Biol Chem* 1998; **273**: 8545–8548.
- 47 Lee DH, Goldberg AL. Proteasome inhibitors: valuable new tools for cell biologists. *Trends Cell Biol* 1998; **8**: 397–403.
- 48 Petit PX, Susin SA, Zamzami N, Mignotte B, Kroemer G. Mitochondria and programmed cell death: back to the future. *FEBS Lett* 1996; **396**: 7–13.
- 49 Slee EA, Zhu H, Chow SC, MacFarlane M, Nicholson DW, Cohen GM. Benzylloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD.FMK) inhibits apoptosis by blocking the processing of CPP32. *Biochem J* 1996; **315**: 21–24.
- 50 Srinivasula SM, Ahmad M, Fernandes-Alnemri T, Alnemri ES. Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. *Mol Cell* 1998; **1**: 949–957.
- 51 Fernandes-Alnemri T, Armstrong RC, Krebs J, Srinivasula SM, Wang L, Bullrich F, Fritz LC, Trapani JA, Tomaselli KJ, Litwack G, Alnemri ES. *In vitro* activation of CPP32 and Mch3 by Mch4, a novel human apoptotic cysteine protease containing two FADD-like domains. *Proc Natl Acad Sci USA* 1996; **93**: 7464–7469.
- 52 MacFarlane M, Cohen GM, Dickens M. JNK (c-Jun N-terminal kinase) and p38 activation in receptor-mediated and chemically-induced apoptosis of T-cells: differential requirements for caspase activation. *Biochem J* 2000; **348**: 93–101.
- 53 Scaffidi C, Medema JP, Kramer PH, Peter ME. FLICE is predominantly expressed as two functionally active isoforms, caspase-8/a and caspase-8/b. *J Biol Chem* 1997; **272**: 26953–26958.
- 54 Bratton SB, Walker G, Roberts DL, Cain K, Cohen GM. Caspase-3 cleaves Apaf-1 into an ~30 kDa fragment that associates with an inappropriately oligomerized and biologically inactive ~1.4 MDa apoptosome complex. *Cell Death Differ* 2001; **8**: 425–433.
- 55 Slee EA, Harte MT, Kluck RM, Wolf BB, Casiano CA, Newmeyer DD, Wang HG, Reed JC, Nicholson DW, Alnemri ES, Green DR, Martin SJ. Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. *J Cell Biol* 1999; **144**: 281–292.
- 56 Freathy C, Brown DG, Roberts RA, Cain K. Transforming growth factor-beta(1) induces apoptosis in rat FaO hepatoma cells via cytochrome c release and oligomerization of Apaf-1 to form a ~700 kDa apoptosome caspase-processing complex. *Hepatology* 2000; **32**: 750–760.
- 57 Qin H, Srinivasula SM, Wu G, Fernandes-Alnemri T, Alnemri ES, Shi Y. Structural basis of procaspase-9 recruitment by the apoptotic protease-activating factor 1. *Nature* 1999; **399**: 549–557.
- 58 Zhou P, Chou J, Olea RS, Yuan J, Wagner G. Solution structure of Apaf-1 CARD and its interaction with caspase-9 CARD: a structural basis for specific adaptor/caspase interaction. *Proc Natl Acad Sci USA* 1999; **96**: 11265–11270.
- 59 Rodriguez J, Lazebnik Y. Caspase-9 and APAF-1 form an active holoenzyme. *Genes Dev* 1999; **13**: 3179–3184.
- 60 Stennicke HR, Deveraux QL, Humke EW, Reed JC, Dixit VM, Salvesen GS. Caspase-9 can be activated without proteolytic processing. *J Biol Chem* 1999; **274**: 8359–8362.
- 61 Bratton SB, Walker G, Srinivasula SM, Sun XM, Butterworth M, Alnemri ES, Cohen GM. Recruitment, activation and retention of caspases-9 and -3 by Apaf-1 apoptosome and associated XIAP complexes. *EMBO J* 2001; **20**: 998–1009.