Heat-shock protein 70 antisense oligomers enhance proteasome inhibitor-induced apoptosis

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Recent evidence supports a role for heat-shock protein 70 (hsp70) and the 26 S proteasome in regulating apoptosis, although the precise nature of their involvement is not known. In the present study, control and *Bcl-x_L*-overexpressing, interleukin-3-dependent FL5.12 cell lines were treated with the proteasome inhibitor *N*-benzoyloxycarbonyl (Z)-Leu-Leu-leucinal (MG132). Basal proteasome activity appeared to be $\approx 30\,\%$ lower in *bcl-x_L* cells compared with control cells using a substrate for the chymotrypsin-like activity. However, no difference in proteasome activity was detected using substrates for the trypsin-like or peptidylglutamyl peptide-hydrolysing activities. In addition, protein levels of the 20 S proteasome β-subunit, as determined by Western blot analyses, were similar in control and *bcl-x_L* cells, leading to the conclusion that proteasome activities were the same in these two cell lines. At 24 h after treatment with 500 nM MG132, apoptosis in *bcl-x_L* cells (22 %) was less than that observed in control cells (34 %). Concomitantly, caspase activity in control cells, as assessed by *N*-acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartyl-7-amino-4-methylcoumarin (Ac-DEVD-AMC), was twice that observed in *bcl-x_L* cells. By 48 h after MG132 treatment, apoptosis and caspase activity in *bcl-x_L* cells were similar to those observed in control cells at 24 h. Proteasome inhibition stimulated increases in hsp70 protein levels in control and *bcl-x_L* cells by 12 h, although the maximal increases found in *bcl-x_L* cells were less. Blocking this induction with hsp70 antisense oligonucleotides potentiated apoptosis after treatment with MG132. Inhibiting caspase activity with a broad-spectrum caspase inhibitor, t-butoxycarbonyl-Asp(OMe)-fluoromethyl ketone, prevented MG132-induced apoptosis. The more specific caspase-3 inhibitor, Ac-DEVD-aldehyde, afforded less protection, although both inhibitors completely inhibited Ac-DEVD-AMC cleavage. These data indicate that both hsp70 and Bcl-x_L provide some protection against proteasome inhibitor-induced apoptosis.

Key words: Bcl-x_L, caspase, MG132.

INTRODUCTION

Initial apoptosis research focused heavily on oligonucleosomal DNA fragmentation as the hallmark of this form of cell death. Recently, however, the role of proteolytic events has taken centre stage. Abundant evidence suggests that activation of a growing family of cysteine–aspartate proteases (caspases) may be the common, unifying thread of cells undergoing apoptosis. In addition, recent evidence suggests a role for the ubiquitin-proteasome pathway in the process of cell death [1]. It may be that the ubiquitin-proteasome pathway is complementary to caspase activation in certain cell types insofar as it acts upstream of caspases, actually stimulating their activation. Alternatively, it could be that inhibition of the ubiquitin-proteasome pathway acts both in parallel with and separate from the caspase cascade and, therefore, represents a novel pro-apoptotic pathway.

The 26 S proteasome is a multisubunit complex that recognizes and degrades, in an ATP-dependent manner, various proteins, including polyubiquitinated, misfolded and short-lived regulatory proteins. This proteasome is found in the cytosol, nucleus and endoplasmic reticulum, and consists of one catalytic 20 S and two regulatory 19 S subunits. The entire protease complex has a sedimentation coefficient of 26 S, and has at least three distinctive proteolytic activities [2,3]. A recent study by An et al. [4] reports that the pro-apoptotic effect of proteasome inhibition relates to the degree to which its chymotrypsin-like activity has been suppressed. Most substrates targeted for degradation by proteasome are polyubiquitinated, involving a complex series of events [1,5]. The precise role of the ubiquitin-proteasome pathway in regulating apoptosis is unclear, but seems to be largely cell-type-specific and/or cell-cycle-specific. Specifically, it appears that the inhibition of proteasome has an anti-apoptotic effect in terminally differentiated or non-rapidly dividing cells, and a pro-apoptotic effect in rapidly dividing cells [6–9].

bcl-2 was the first mammalian homologue of the *ced* gene family to be discovered, and its corresponding protein improves cell survival following numerous pro-apoptotic stimuli. Additional proteins, both pro- and anti-apoptotic, with sequence similarity to Bcl-2 have since been identified. Taken together, these proteins make up the *bcl-2* family of proto-oncogenes [10]. The mechanism by which each of these proteins exerts its pro- or anti-apoptotic effect is not entirely clear, although recent work with Bax has suggested an important role in regulating mitochondrial permeability [11,12].

Heat-shock protein 70 (hsp70) is a 72-kDa stress protein that, like Bcl-2 and Bcl-x_L, can block apoptosis that is induced by a variety of initiators [13–15]. hsp70 expression is stimulated by the accumulation of abnormal proteins [16], and this protein has

Abbreviations used: hsp, heat-shock protein; IL, interleukin; AMC, 7-amino-4-methylcoumarin; Ac-DEVD, *N*-acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartyl; Boc-D-FMK, t-butoxycarbonyl-Asp(OMe)-fluoromethyl ketone (OMe is methyl ester); NHMec, aminomethyl coumarylamide; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase; Nap, naphthylamide; Z, *N*-benzoyloxycarbonyl.

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been suggested to rescue cells from apoptosis at a late stage in the overall process [17]. The cytoprotective mechanism of hsp70 is likely to involve its chaperone activity with the folding, assembly and degradation of proteins. One recent study indicates that hsp70-mediated cytoprotection involves the inhibition of caspase activation [14]. These authors also determined that heat-induced apoptosis correlated with an increase in stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) activity. Overexpression of hsp70 prevented stress-induced (heat stress and ceramide) apoptosis by interfering with events both upstream and downstream of the SAPK/JNK signalling pathway, which might be associated with caspase activation. Moreover, Gabai et al. [18] showed that incubation of PEER cells with amino acid analogues that disrupt newly synthesized proteins stimulates JNK activation, which is blocked by hsp70 overexpression. This suggests that hsp70 acts as a 'sensor' of abnormal or aberrant proteins that are able to stimulate JNK activity.

The present study examined the ability of hsp70 and *Bcl-x_L* overexpression to protect against proteasome inhibitor-induced apoptosis in FL5.12 cells, an interleukin (IL)-3-dependent murine pro-B lymphocytic cell line. The results indicate that *Bcl-x_L* overexpression delays, but ultimately does not protect against, proteasome inhibitor-induced apoptosis. Proteasome inhibition stimulated caspase activation and hsp70 production in both cell lines, although to a greater extent in control cells, at 24 h. Blocking proteasome-inhibitor-induced increases in hsp70 protein with hsp70 antisense oligonucleotides significantly enhanced apoptosis, suggesting that hssp70 has an important protective role in this system.

EXPERIMENTAL

Cell culture and proteasome inhibition

FL5.12 cells transfected to stably express either an SFFV-NEO control construct or an SFFV-FLAG-*Bcl-x_L* construct were used in these studies, and have been described previously [19]. Cells were cultured in RPMI 1640 complete medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Summit Biotechnology, Fort Collins, CO, U.S.A.), 1% (w/v) glutamine, streptomycin and penicillin (100 μg/ml and 100 units/ml respectively) and 10% (v/v) IL-3-conditioned medium derived from WEHI-3B cells [20]. Both FL5.12 and WEHI-3B cells were maintained in an air/CO_2 (19:1) atmosphere at 37 °C. For proteasome inhibition, cells (10^6/ml) were plated in vented 50-ml flasks (Falcon; Lincoln Park, NJ, U.S.A.) and treated with DMSO [0.1% (v/v) final concentration] as vehicle control, Z-Leu-Leu-leucinal (MG132; a peptide aldehyde inhibitor of proteasome, where Z is *N*-benzoyloxycarbonyl) or lactacystin (a *Streptomyces* metabolite that acts as a proteasome inhibitor) for 8, 12, 24 or 48 h (see the Results section for the concentrations of the latter two compounds). In some cases, cells were treated with *N*-acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartyl (Ac-DEVD)-aldehyde (Research Biochemicals International, Natick, MA, U.S.A.) or t-butoxycarbonyl-Asp(OMe)-fluoromethyl ketone (Boc-D-FMK; Enzyme Systems, Livermore, CA, U.S.A.) before MG132 treatment to inhibit caspase activity. Cell counts were determined using a T-890 Coulter counter (Coulter, Miami, FL, U.S.A.).

Assay of 26 S proteasome proteolytic activities

Control and *bcl-x_L* (*Bcl-x_L*-overexpressing) cells (10^6) were lysed in 30 μl of ice-cold buffer [20 mM Tris/HCl (pH 7.2)/0.1 mM EDTA/1 mM 2-mercaptoethanol/5 mM ATP/20% (v/v) glycerol/0.04% (v/v) Nonidet P40] by repeated pipetting, followed by a 20 min incubation on ice. Cell lysates were centrifuged at 16000 ***g*** for 10 min, and protein concentrations in the resulting supernatants were then measured by the method of Bradford [21]. Total protein (10 μg) was added to 50 mM Hepes, pH 8.0, containing 5 mM EGTA. Substrates for 26 S proteasome (all obtained from Sigma, St. Louis, MO, U.S.A.) dissolved in DMSO specific for the chymotrypsin-like activity [Leu-Leu-Val-Tyr-aminomethyl coumarylamide (LLVY-NHMec)] (50 μM), trypsin-like activity [Leu-Ser-Thr-Arg (LSTR)-NHMec] (50 μM) or peptidylglutamyl peptide-hydrolysing activity [Z-Leu-Leu-Glu (LLE) naphthylamide (Nap)] (500 μM) were added to the mixture and incubated at 37 °C for 30 min [22]. The total volume of cell protein, Hepes buffer and proteasome substrate amounted to 200 μl, and the reaction was performed in a 4-ml disposable test tube at 37 °C. Reactions were started by the addition of proteasome substrate. At the end of a 30-min incubation, 2 ml of water was added to each sample containing either LLVY-NHMec or LSTR-NHMec. Fluorescence was measured using excitation and emission wavelengths of 370 and 430 nm respectively. In the case of Z-LLE-Nap as substrate, the reaction was stopped by adding 0.3 ml of ethanol. Water (2 ml) was then added before measuring fluorescence ($\lambda_{excitation}$ 333 nm, $\lambda_{emission}$ 450 nm).

Lysosomal β-hexosaminidase activity

Control and *bcl-x_L* cells (10^6) were suspended in 50 μl of ice-cold buffer [10 mM Tris/HCl (pH 7.4)/140 mM NaCl/1% (v/v) Triton X-100/4 μg/ml antipain/20 μg/ml benzamidine/2 μg/ml each of leupeptin, pepstatin and chymostatin], and then sonicated for 2 min. Aliquots (10 μg of total protein) of the extract were added to 100 mM citrate/phosphate buffer, pH 4.5, and mixed with *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide (4 mM final concentration). After 30 min at 37 °C, the reaction was stopped by adding 250 μl of 0.08 M NaOH, and water was added to bring the volume to 1 ml. The absorbance of the *N*-acetyl-*p*-nitrophenol formed was determined at 410 nm.

Apoptosis measurements

Apoptosis was quantified using the ethidium bromide and Acridine Orange assay [23]. Briefly, control and *bcl-x_L* cells (10^6) were transferred to microcentrifuge tubes and pelleted for 10 min at 200 ***g***. After centrifugation, the cell-rich pellets were resuspended in a mixture containing 2 μl of ethidium bromide (100 μg/ml) and 2 μl of Acridine Orange (100 μg/ml). Live, apoptotic, secondary necrotic and necrotic cells were differentiated from each other using fluorescence microscopy on the basis of definitive nuclear and cytosolic fluorescence and distinct morphological changes, including membrane blebbing, nuclear and cytosolic condensation, and nuclear fragmentation. A minimum of 150 cells were counted in four random fields per slide.

DNA fragmentation as oligonucleosomal ladder formation was assessed as described previously [24] using control and *bcl-x_L* cells (5×10^6) treated with 500 nM MG132 for 24 h.

Phosphatidylserine exposure on the plasma membrane was measured directly by the binding of annexin V–FITC to phosphatidylserine in the presence of Ca^{2+} using the annexin V FITC kit (Immunotech, Miami, FL, U.S.A.). Cells were also stained with propidium iodide (100 μg/ml) to differentiate between necrotic and non-necrotic cell populations. The cells were analysed with a Coulter EPICS-XL flow cytometer equipped with an argon laser. Histograms were constructed for 10000 cells in order to determine the percentage of apoptotic cells showing positive annexin V–FITC binding and those permeable to propidium iodide.

A characteristic event in apoptosis is DNA fragmentation and release of nucleosomes into the cytoplasm. These can be detected by an ELISA assay that is quite sensitive and specific for apoptosis relative to necrosis [25]. Cells were gently lysed, releasing mono- and oligo-nucleosomes from apoptotic cells. The assay is completed by a one-step ELISA procedure, the kit (Roche Molecular, Indianapolis, IN, U.S.A.) for which comprises streptavidin-coated microplates, biotinylated anti-histone and peroxidase-conjugated anti-DNA antibodies. Results are expressed as the $\Delta A_{405-490}$, which indicates the enrichment of nucleosomes in the cytoplasm.

Measurement of caspase activity

Cells (10^6) were pelleted at 200 ***g*** for 10 min, and lysed on ice for 10 min in 200 μl of buffer [50 mM Tris/HCl (pH 7.5)/150 mM NaCl/0.5 mM EDTA/0.5 % (v/v) Nonidet P40]. Aliquots o cell lysates (50 μl) were added to microtitre plates, together with 140 μl of assay buffer [10 mM Hepes (pH 7.5)/50 mM NaCl/2.5 mM dithiothreitol] and 40 μM Ac-DEVD-[7-amino-4-methylcoumarin (Ac-DEVD-AMC)]. Caspase-3 possesses optimal substrate specificity for the tetrapeptide sequence DEXD [26]. This sequence is also a substrate for caspases-2 and -7. Cleavage of DEVD releases free AMC, which was measured using an excitation wavelength of 365 nm and emission wavelength of 450 nm [27].

Western blot assays

Pelleted cells (5×10^6) were resuspended and lysed in 100 μl of ice-cold lysis buffer [10 mM Tris/HCl (pH 7.4)/10 mM NaCl/3 mM $MgCl_2$/1 mM EDTA/0.1 % (v/v) Nonidet P40/100 μg/ml PMSF/30 μl/ml aprotinin/1 mM sodium orthovanadate] by repeated pipetting, followed by a 15-min incubation on ice. Cell lysates were centrifuged at 16000 ***g*** for 10 min, and the supernatant was collected and stored at -20 °C. Protein concentrations of thawed supernatants were determined [21], and aliquots were mixed with loading dye [4 % (w/v) SDS/20 % (w/v) glycerol/4 % (w/v) 2-mercaptoethanol/0.2 M Tris/HCl (pH 6.8)/0.02 % (w/v) Bromophenol Blue] and electrophoresed on SDS/polyacrylamide reducing gels (polyacrylamide concentration either 8 % for hsp70 or 15 % for pro-caspase-3 or the 20 S proteasome β-subunit). Protein was transferred to PVDF membranes (Millipore, Bedford, MA, U.S.A.) and blocked for at least 2 h in 5 % (w/v) non-fat dry milk (BioRad, Hercules, CA, U.S.A.). Membranes were then incubated with a polyclonal antibody specific for the inducible form of hsp70 (1:20000 dilution) (StressGen, Victoria, BC, Canada), a polyclonal anti-(pro-caspase-3) antibody (1:500 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), or polyclonal antiserum raised against the 20 S proteasome β-subunit (1:5000 dilution) (Calbiochem, La Jolla, CA, U.S.A.) for 1 h. The membranes were rinsed and incubated with a horseradish-peroxidase-conjugated secondary antibody (1:3000 dilution) (Amersham, Arlington Heights, IL, U.S.A. and Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) in 2 % (w/v) non-fat dry milk for 1 h. After the secondary antibody incubation, the membranes were rinsed and bound antibodies were detected using enhanced chemiluminescence with a kit from Amersham. Individual band densities were integrated following scanning using NIH Image public domain software.

L-Canavanine treatment

Cells (10^6/ml) were cultured for 4 h in complete RPMI 1640 medium, except that L-arginine was replaced by its analogue L-canavanine (10 mM). At the end of 4 h, cells were centrifuged at 200 ***g***, the supernatant was removed and the cell-rich pellet was resuspended in complete RPMI 1640 medium with L-arginine for 8 h. At this time, cells were processed for apoptosis measurements, Western blot detection of hsp70 and/or treatment with 500 nM MG132 for 24 h.

hsp70 antisense treatment

Cells (10^6/ml) were plated in Opti-MEM I reduced-serum medium (Gibco, Gaithersburg, MD, U.S.A.) containing 6.6 μl of Lipofectin Reagent (Gibco). Optimized antisense oligomers (5′-CACCTTGCCGTGCTGGAA-3′) (10 μM), customized on the basis of the coding region (nt no. 61–78) of the mouse heat-inducible *hsp70* gene (Oligos Etc., Wilsonville, OR, U.S.A.) [28], were added to the cells and allowed to incubate for 4 h at 37 °C. At that time, an equal amount of complete growth medium (described above) was added to the cells, followed by an additional 20-h incubation. Then, the cells were centrifuged at 200 ***g*** for 10 min and replated in fresh Opti-MEM medium containing Lipofectin and 10 μM antisense oligomers. After 90 min, an equal volume of complete growth medium was added to the cells and experiments begun. Control experiments using nonsense oligonucleotides (5′-TGGATCCGACATGTCAGA-3′) were run concurrently.

Statistics

Data are expressed as means $\pm$ S.E.M. Comparisons between groups were done using analysis of variance followed by a *post-hoc* analysis using Student–Newman–Keul's test. A P value of < 0.05 was considered to be significant.

RESULTS

Proteasome activities

26 S proteasome chymotrypsin-like activity was measured using the peptide substrate LLVY-NHMec. Surprisingly, baseline activity was ≈ 30 % higher in vehicle-treated control versus *bcl-x_L* cells (Table 1). Analyses using substrates relatively specific for the trypsin-like (LSTR-NHMec) or peptidylglutamyl peptide-hydrolysing (Z-LLE-Nap) proteasome activities revealed no differences between control and *bcl-x_L* cells (Table 1). In addition, 20 S proteasome β-subunit protein levels, determined by Western blot analyses, were identical in these two cell lines (Figure 1). Lysosomal β-hexosaminidase activity was slightly, but not significantly, less in *bcl-x_L* cells (relative activities of 3.8 ± 0.2 and 4.4 ± 0.3 respectively). It appears, therefore, that proteasome content and activity are the same in control and *bcl-x_L* cells. The difference observed with the LLVY substrate may be related to cross-reactivity with lysosomal cysteine proteases. Treatment for 3 h (a time at which no apoptosis was observed) with 500 nM MG132 or 1 μM lactacystin caused a similar (approx. 80–90 %) inhibition of the chymotrypsin-like proteasome activity in control and *Bcl-x_L*-overexpressing cells (Table 1).

MG132- and lactacystin-induced apoptosis

Initial dose–response experiments using control FL5.12 cells revealed no increase in apoptosis above baseline levels by 24 h (as assessed by Acridine Orange/ethidium bromide staining) with doses up to 100 nM MG132. Doses of 250 nM, 500 nM and 1 μM caused respectively ≈ 20, 33 and 78 % apoptosis by 24-h post-MG132. To verify that this pro-apoptotic effect was due to proteasome inhibition, a dose–response experiment was performed with the more specific 26 S proteasome inhibitor,

Table 1 26 S proteasome activities

LLVY-NHMec (50 μM), LSTR-NHMec (50 μM) and Z-LLE-Nap (500 μM) were added to an incubation mixture containing 10 μg of cellular protein and incubated as described in the Experimental section. The inhibitor studies were performed on cells incubated with 500 nM MG132 or 1 μM lactacystin for 3 h at 37 °C before lysis. Results are expressed as mean fluorescence units ± S.E.M. ($n = 3$). *, Significantly less than basal activity ($P < 0.05$).

(a)

Treatment	26 S proteasome activity (units)	
	Control cells	*bcl-x*$_L$ cells
Chymotrypsin-like substrate (LLVY-NH-Mec)	85.9 ± 4.3	58.3 ± 3.8
Trypsin-like substrate (LSTR-NH-Mec)	11.6 ± 0.1	11.8 ± 0.9
Peptidylglutamyl peptide-hydrolysing substrate (Z-LLE-Nap)	4.9 ± 0.2	4.4 ± 0.4

(b)

Inhibition of chymotrypsin-like activity	26 S proteasome activity (units)	
	Control cells	*bcl-x*$_L$ cells
MG132	17.9 ± 0.2*	13.2 ± 1.3*
Lactacystin	7.1 ± 1.1*	4.8 ± 1.4*

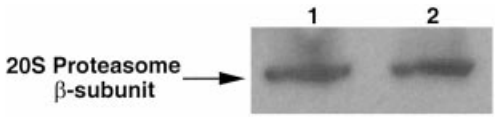

Figure 1 Western blot analysis of 20 S proteasome β-subunit protein levels in FL5.12 cells

A Western blot was performed on cell extracts from control (lane 1) and *Bcl-x*$_L$-overexpressing (lane 2) cells. Each lane was loaded with 50 μg of total protein and analysed by SDS/PAGE (15% gels).

Table 2 Apoptosis following treatment with MG132

Control and Bcl-x$_L$-overexpressing FL5.12 cells (10^6/ml) were plated in fresh complete medium and treated with either DMSO or 500 nM MG132 for up to 24 h. Data are expressed as the percentage of total cells that were apoptotic, which was determined using distinct nuclear and cytosolic fluorescence in the presence of Acridine Orange/ethidium bromide. A minimum of 150 cells in 4 random fields were counted per slide. Results are expressed as means ± S.E.M ($n = 3$). *, Significantly different from untreated counterpart at the same time ($P < 0.05$).

Time (h)	Control cells		*bcl-x*$_L$ cells	
	Vehicle	+ MG132	Vehicle	+ MG132
0	0.80 ± 0.15	0.83 ± 0.09	0.94 ± 0.10	0.79 ± 0.11
8	1.33 ± 0.03	2.06 ± 0.17	0.65 ± 0.18	0.83 ± 0.19
12	0.87 ± 0.44	12.8 ± 1.5*	1.23 ± 0.14	5.36 ± 0.86*
24	3.37 ± 0.35	34.0 ± 1.5*	2.4 ± 0.3	21.9 ± 1.4*

lactacystin. A slight increase in apoptosis above the baseline was observed with 500 nM lactacystin by 24 h. Lactacystin at concentrations of 1 and 10 μM caused $\approx$ 15 and $\approx$ 60% apoptosis respectively, measured at the same time point. Furthermore, 10 μM lactacystin produced $\approx$ 20% secondary necrosis by 24 h, whereas 1 μM lactacystin yielded less than 5% secondary necrosis.

On the basis of these experiments, a final concentration of 500 nM MG132 was used for all subsequent studies. By 24 h after plating, the level of apoptosis in vehicle-treated cells was approx. 3%, as determined by Acridine Orange/ethidium bromide staining (Table 2). No significant increase in apoptosis was evident in either cell line at 8 h post-MG132 treatment. At 12 h after MG132, apoptosis in control and *bcl-x*$_L$ cells had increased to $\approx$ 13% and 5% of total cells respectively (Table 2). This increase continued to 24 h in both control and *bcl-x*$_L$ cells, when apoptotic cells represented $\approx$ 34% and 22% of total cell counts respectively (Table 2). At 48 h, the percentage of apoptotic *bcl-x*$_L$ cells measured by Acridine Orange/ethidium bromide staining had increased to $\approx$ 32%. Control MG132-treated cells were not examined at this time-point because of poor overall viability. Necrotic cells in both cell lines never accounted for more than 7% of total cell counts up to 24 h.

The apoptosis induced by MG132 was confirmed by finding significant oligonucleotide cleavage in the form of a DNA ladder in control cells 24 h after treatment with MG132 (results not shown). Only faint DNA laddering was visible in Bcl-x$_L$ overexpressors at 24 h, whereas a more visible DNA ladder was present in *bcl-x*$_L$ cells by 48 h after treatment with 500 nM MG132, when apoptosis levels had increased as shown by the Acridine Orange/ethidium bromide data.

Changes in hsp70 levels

Western-blot data revealed similar constitutive levels of the inducible form of hsp70 in both control and *Bcl-x*$_L$ overexpressing cell lines (Figure 2). There were increases in hsp70 levels to approx. 2.5- and 1.5-fold above baseline in control and *Bcl-x*$_L$-overexpressing cells respectively by 12 h post-MG132 treatment (Figure 2). By 24 h post-MG132, hsp70 levels had declined in both control and *bcl-x*$_L$ cells (probably due to increased apoptosis and/or the half-life of this protein).

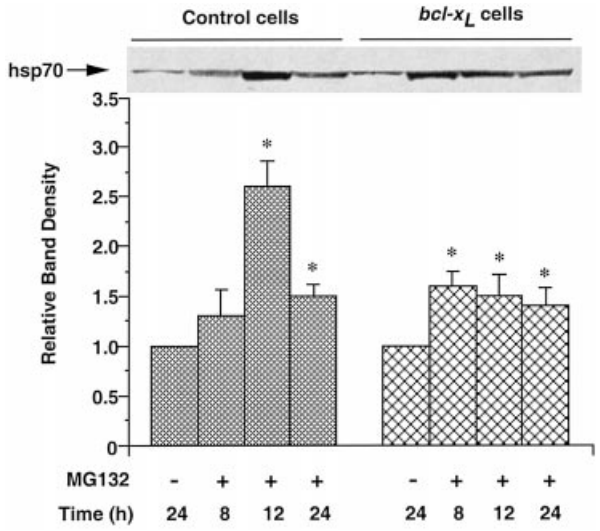

Figure 2 Western blot analysis of hsp70 protein levels in FL5.12 cells treated with MG132

Cells were vehicle-treated or treated with 500 nM MG132 for 8, 12 and 24 h. Cells were lysed, and the lysate was used to perform SDS/PAGE as described in the Experimental section. Each lane was loaded with 30 μg of total protein, and a primary antibody specific for the inducible form of hsp70 was used. A representative Western blot is shown. The histogram shows the quantitative analyses of density data from three independent blots. Data are expressed as means $\pm$ S.E.M. *, Significantly different from cells not treated with MG132 ($P < 0.05$).

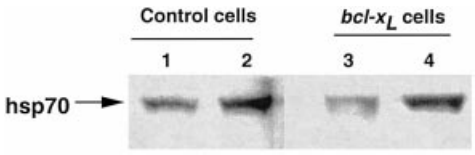

Figure 3 Western blot analysis of hsp70 protein levels in FL5.12 cells treated with L-canavanine

Cells were vehicle-treated or treated with L-canavanine-rich medium for 4 h, followed by an 8 h recovery in complete IL-3 medium. Lanes 1 and 3 represent vehicle-treated control and *bcl-x$_L$* cells respectively. Lanes 2 and 4 represent L-canavanine-treated control and *bcl-x$_L$* cells respectively. Each lane was loaded with 30 μg of total protein.

L-Canavanine and antisense experiments

To determine what, if any, effect increasing levels of hsp70 had on MG132-induced apoptosis, cells were treated with the L-arginine analogue L-canavanine to induce hsp70. Incubating control and *bcl-x$_L$* cells in L-arginine-deficient culture medium supplemented with 10 mM L-canavanine for 4 h, followed by an 8 h recovery in complete IL-3-containing growth medium, stimulated a 1.7- to 2-fold (as determined by densitometry) increase in hsp70 levels compared with untreated cells (Figure 3; cf. lanes 1 and 2, and lanes 3 and 4). This treatment resulted in no apoptosis above untreated levels, as measured by Acridine Orange/ethidium bromide staining at 24 h. However, elevated levels of hsp70, induced by L-canavanine, afforded no protection against MG132-induced apoptosis at 24 h post-treatment in either control or *bcl-x$_L$* transfectants (results not shown).

To determine whether decreasing hsp70 levels affected proteasome inhibitor-induced apoptosis, control cells were pretreated with 10 μM antisense oligonucleotides of the mouse heat-inducible *hsp70* gene for a total of 25.5 h prior to a subsequent

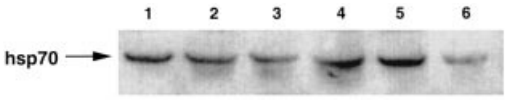

Figure 4 Western blot analysis of hsp70 after treatment of FL5.12 cells with hsp70 antisense oligonucleotide

Control FL5.12 cells were treated with 10 μM nonsense or 10 μM antisense oligonucleotides based on the coding region (nt. nos. 61–78) of the mouse heat-inducible *hsp70* gene in the presence or absence of 500 nM MG132. Specific antisense and nonsense treatments before treatment with MG132 are described in the Experimental section. Lane 1, vehicle-treated; lane 2, nonsense alone; lane 3, antisense alone; lane 4, nonsense + MG132; lane 5, MG132 alone; lane 6, antisense + MG132. Each lane was loaded with 30 μg of total protein.

500 nM MG132 treatment for 24 h. The presence of antisense oligonucleotide alone led to a modest decline in constitutive hsp70 protein levels (Figure 4, cf. lanes 3 and 1). However, MG132-induced increases in hsp70 protein were completely blocked by hsp70 antisense oligonucleotide at 8 h post-MG132, as evidenced by the 3.5-fold difference (determined by densitometry) in the levels of this protein between control cells treated with MG132 alone and those cells pretreated with antisense oligonucleotides (Figure 4, cf. lanes 5 and 6). Pretreatment of control cells with 10 μM nonsense oligonucleotides had no effect on either constitutive or MG132-induced hsp70 protein levels (Figure 4, cf. lanes 2 and 4 with lane 1).

Pretreatment of control cells with antisense oligonucleotide had a potentiating effect on MG132-induced apoptosis by 24 h, as measured by Acridine Orange/ethidium bromide staining. Specifically, 10 μM hsp70 antisense oligonucleotide resulted in $\approx 64\%$ apoptosis in MG132-treated cells compared with $\approx 36\%$ apoptosis in cells that were pretreated with 10 μM nonsense oligonucleotide and those treated with MG132 alone. Moreover, $\approx 22\%$ of control cells pretreated with hsp70 antisense oligomers were secondarily necrotic by 24 h, leaving only $\approx 14\%$ live cells. Secondary necrosis accounted for less than 15% of the total cell counts in both nonsense pretreated and MG132-treated alone cells at the same time point. The morphology of untreated control cells (Figure 5A) and those treated with MG132 alone (Figure 5B) or in the presence of either 10 μM nonsense (Figure 5C) or 10 μM antisense (Figure 5D) oligonucleotides is represented.

Acridine Orange/ethidium bromide apoptosis data were confirmed by annexin V–FITC and propidium iodide co-treatment analysed by flow cytometry. At 12 h, $\approx 7\%$ of cells pretreated with hsp70 antisense oligomers were apoptotic compared with $\approx 3\%$ of nonsense pretreated and MG132-treated alone cells (results not shown). By 18 h, less than 2% of vehicle-treated control cells were apoptotic (Table 3). In contrast, cells treated with 500 nM MG132 alone and those cells pretreated with 10 μM nonsense oligonucleotide exhibited $\approx 25\%$ apoptosis and $\approx 12\%$ secondary necrosis (Table 3). Cells pretreated with 10 μM antisense oligonucleotide showed $\approx 37\%$ apoptosis and $\approx 17\%$ secondary necrosis (Table 3).

Activation of caspase-3

Similar levels of pro-caspase-3 protein were detected in cell lysates from vehicle-treated control and *bcl-x$_L$* cells (Figure 6A, lanes 1 and 5). Levels of this protein in control cells began to decline by 8 h after 500 nM MG132 treatment (lane 2), and were 30% of vehicle-treated values by 24 h (lane 4). In contrast, there was no decline in pro-caspase-3 levels in *Bcl-x$_L$* overexpressors

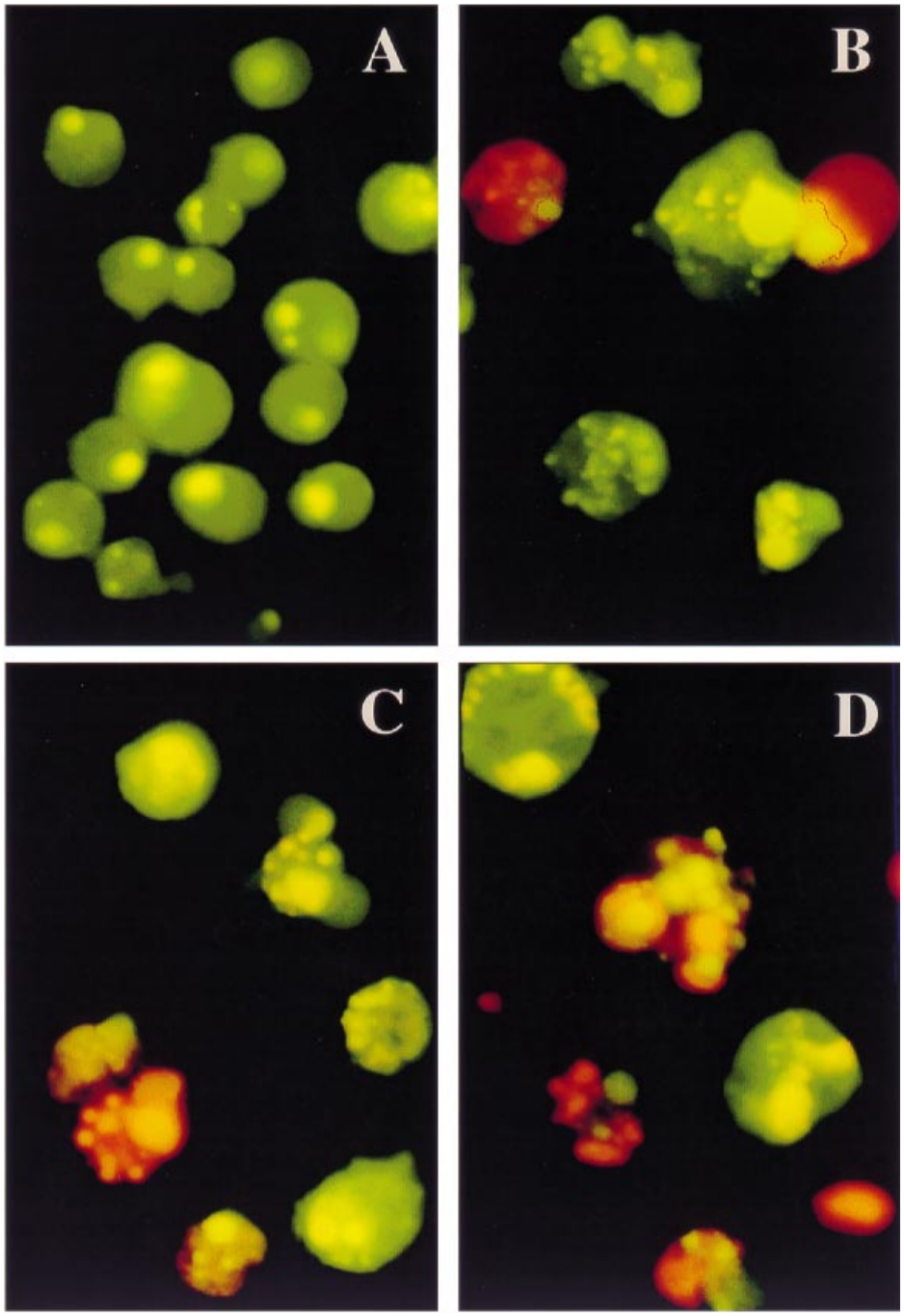

Figure 5 Morphology of control FL5.12 cells using Acridine Orange/ethidium bromide staining

Cells were treated with either 10 μM nonsense or 10 μM antisense oligonucleotides for 25.5 h followed by treatment with 500 nM MG132 for 24 h. Treatments were: (**A**) DMSO (vehicle); (**B**), MG132 alone; (**C**) nonsense + MG132; and (**D**) antisense + MG132. Live cells stain green and are round with two or fewer yellow dots; apoptotic cells with intact plasma membranes stain green, are irregularly shaped, often due to membrane blebbing, and contain multiple yellow/green dots of condensed nuclei. Cells undergoing secondary necrosis stain bright orange due to the influx of ethidium bromide, and contain multiple yellow/green/orange condensed nuclei. Magnification = $\times$360.

until 12 h (lane 7). These diminished levels ($\approx 35\%$ below vehicle-treated) were then maintained up to 24 h (lane 8).

In order to quantify caspase activity in cell extracts, the release of the fluorochrome AMC from the caspase substrate, Ac-DEVD-AMC, was measured (Figure 6B). No significant increase above untreated levels of Ac-DEVD-AMC cleavage was detected in either cell line up to 8 h post-MG132 treatment. A slight, but significant, increase in Ac-DEVD-AMC cleavage was detected in control cells, but not in *bcl-x*$_L$ cells, at 12 h. By 24 h, $\approx$ 7- and 3.5-fold increases in Ac-DEVD-AMC cleavage above untreated levels were measured in control and *bcl-x*$_L$ cells respectively. By 48 h after MG132 treatment, Ac-DEVD-AMC cleavage in *bcl-x*$_L$ cells had increased to levels observed in control MG132-treated cells at 24 h (Figure 6B).

Table 3 Apoptosis in cells treated with *hsp70* antisense oligonucleotides

Control cells (10^6/ml) were treated with either 0.1% DMSO (vehicle), 500 nM MG132 alone, 10 μM nonsense + MG132, or 10 μM antisense + MG132 for 18 h. Cells were co-stained with annexin V–FITC and propidium iodide and analysed by flow cytometry as described in the Experimental section.

Treatment	Condition...	Proportion of cells (%)		
		Live	Apoptotic	Secondarily necrotic
Vehicle		94.6	1.2	2.4
MG132		58.6	25.4	10.4
Nonsense + MG132		58.5	24.8	12
Antisense + MG132		46.4	36.7	17.2

Table 4 Ac-DEVD-AMC cleavage activity in MG132-treated cells

Control and *bcl-x*$_L$ cells (10^6) were treated with either Ac-DEVD-CHO (25 μM) or Boc-D-FMK (20 μM) for 90 min before MG132 treatment (500 nM). Caspase activity was measured at 24 h in the lysate as described in the Experimental section. As shown in Figure 6(B), caspase activities were the same in vehicle-treated control and *bcl-x*$_L$ cells at 24 h. Results are expressed as mean percentages ± S.E.M. ($n = 3$). *, Significantly different from other treatments ($P < 0.05$).

Treatments	Caspase activity (% of vehicle)	
	Control cells	*bcl-x*$_L$ cells
0.1% DMSO (vehicle)	100 ± 10	100 ± 9
MG132	724 ± 82*	384 ± 58*
MG132 + Ac-DEVD-CHO	21.4 ± 2.9	26.9 ± 3.9
MG132 + Boc-D-FMK	35.7 ± 2.1	42.3 ± 3.8

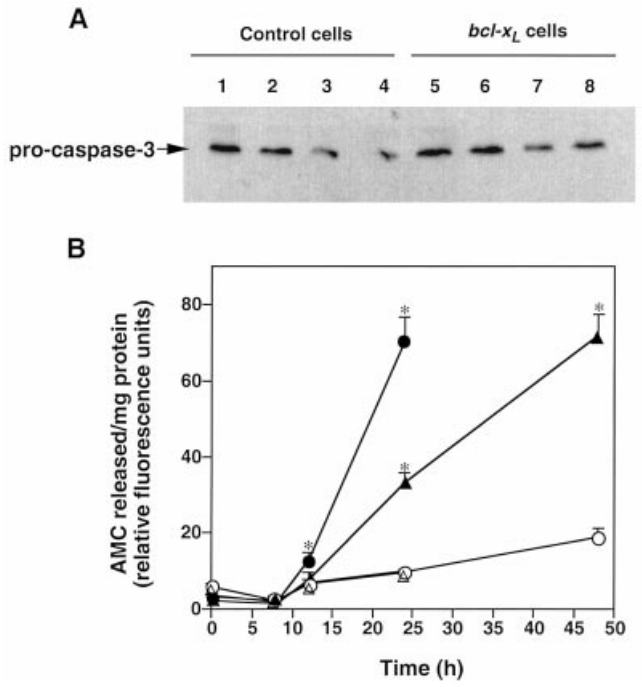

Figure 6 Caspase activation in MG132-treated FL5.12 cells

(**A**) Western blot analysis of pro-caspase-3 in cells treated with 500 nM MG132. The lanes represent the vehicle-treated state (24 h) and 8, 12 and 24 h after treatment with 500 nM MG132 in control (lanes 1–4) and *bcl-x*$_L$ cells (lanes 5–8). Each lane was loaded with 30 μg of total protein. (**B**) Ac-DEVD-AMC cleavage activity in control and *bcl-x*$_L$ cells after treatment with 500 nM MG132. The traces represent vehicle-treated control (○) or *bcl-x*$_L$ cells (△), and MG132-treated control (●) or *bcl-x*$_L$ cells (▲). Cells (10^6) were plated and treated with 500 nM MG132 for 0, 8, 12, 24 or 48 h. Cells were lysed at the different time points, and the lysate was used to measure caspase activity as described in the Experimental section. Results are expressed as means ± S.E.M. ($n = 3$). *, Significantly different from the untreated counterpart ($P < 0.05$).

Effect of caspase inhibitors

Cells were pretreated for 90 min with either 25 μM Ac-DEVD-aldehyde or 20 μM Boc-D-FMK before treatment with 500 nM MG132. This treatment completely inhibited Ac-DEVD-AMC cleavage for 24 h (Table 4), documenting the cellular uptake of these inhibitors. Using both annexin V binding and an ELISA assay that detects oligonucleosome formation, the general caspase inhibitor Boc-D-FMK prevented MG132-induced apoptosis (Table 5). The more specific caspase-3 inhibitor Ac-DEVD-aldehyde was less effective, but still decreased MG132-induced apoptosis (Table 5).

DISCUSSION

Numerous reports have described both the pro- and anti-apoptotic effects of a variety of proteasome inhibitors. In general, inhibition of the 26 S proteasome elicits either an anti-apoptotic effect in terminally differentiated or non-rapidly dividing cells or a pro-apoptotic effect in rapidly dividing cells. Although the mechanism(s) involved in these different responses is/are unknown [29], the inability to dispose of abnormal proteins resulting in the generation of conflicting signals for growth and cell-cycle arrest is considered to be the key in determining apoptotic effects of proteasome inhibitors. The accumulation of such proteins is reflected by the induction of stress proteins, such as hsp70 [30]. MG132 stimulated hsp70 induction in both control and *Bcl-x*$_L$-overexpressing cells up to 24 h post-treatment. Although this increase in hsp70 was observed as early as 8 h post-MG132 (a time at which no apoptosis was detected), the increase was not sufficient to prevent apoptosis. Nevertheless, the ability of antisense oligonucleotides against the inducible form of hsp70 to prevent this induction and potentiate MG132-induced apoptosis suggests that hsp70 is capable of affecting apoptosis in this system. Specifically, these data indicate that hsp70 induction stimulated by MG132 slows the apoptotic response to this stimulus, and blocking this induction results in increased apoptosis.

It has been reported that apoptosis in M-07e cells, induced by granulocyte–macrophage colony-stimulating factor withdrawal, is associated with the loss of Bcl-2 protein, and that this loss is greatly increased by MG132 [31]. It has also been reported that proteasome inhibition has no effect on Bcl-2 levels, but causes a modest down-regulation of Bcl-x$_L$ protein in a prostate carcinoma cell line [32]. The authors noted that the significance of this latter effect was not clear. Although the loss of anti-apoptotic BCL-2 proteins might promote apoptosis, it is apparently not required for the apoptotic activity of MG132, since in FL5.12 cells, which do not lose either Bcl-2 or Bcl-x$_L$ upon withdrawal of IL-3 ([33] and unpublished work), Bcl-x$_L$ and Bax protein levels were not affected by treatment with this proteasome inhibitor (unpublished work).

Protection against apoptosis can be achieved by several endogenous proteins. The most widely studied are the BCL-2 family, with Bcl-2 and Bcl-x$_L$ being the most prominent anti-apoptotic members. hsp70 is also able to protect against apoptosis

Table 5 Effect of caspase inhibitors on MG132-induced apoptosis

Cells (10^6/ml per well) were pretreated with either Boc-D-FMK (30 μM) or Ac-DEVD-aldehyde (25 μM) for 2 h followed by 500 nM MG132. Boc-D-FMK or Ac-DEVD-aldehyde treatment alone did not cause apoptosis. Cells were either co-treated with annexin V–FITC and propidium iodide and analysed by flow cytometry 18 h after treatment with MG132, as described in the Experimental section, or they were analysed 24 h after treatment with MG132. Results were determined as the mean $\Delta A_{405-490} \pm$ S.E.M. relative to control cells, which indicates the enrichment of nucleosomes in the cytoplasm ($n = 3$).

	Annexin V (18 h)		ELISA (24 h)	
	Live (% of total cell count)	Apoptotic (% of total cell count)	$\Delta A_{405-490}$	Enrichment factor
Control	94.3	4.8	0.065 ± 0.005	1.0
MG132	25.7	73.8	0.425 ± 0.041	6.5
MG132 + BOC-D-FMK	90.4	7.6	0.091 ± 0.010	1.4
MG132 + DEVD-aldehyde	66.8	32.4	0.264 ± 0.029	4.1

[13–15]. As for the BCL-2 proteins, the precise molecular cytoprotective mechanism of hsp70 is not understood. In general, the protective mechanism of hsp70 is believed to be similar to that of other heat-shock proteins in that it behaves as a chaperone by preventing the denaturing and misfolding of proteins [30]. Others suggest that the cytoprotective effect of hsp70 might arise from its ability to assist in the transfer of newly synthesized proteins into mitochondria, helping to maintain overall mitochondrial integrity [34], or by preventing apoptosis by inhibiting caspase activity [14]. On the other hand, recent data also suggest hsp70 exerts its anti-apoptotic effect downstream of caspases [17].

Previous studies reported that overexpression of Bcl-2 and Mcl-1, a protein with sequence similarity to Bcl-2, affords protection against heat stress [35–37]. This suggests a potential functional similarity between hsp70 and BCL-2 proteins. Interestingly, the induction of hsp70 by MG132 was attenuated in Bcl-x_L-overexpressing cells. This is similar to our previous findings with hsp70 induction following heat stress in these same cells [15], and suggests that the stimulus for hsp70 induction is diminished in Bcl-x_L-overexpressing cells, perhaps because the BCL-2 proteins affect some of the same pathways as hsp70. Given the pore-forming nature of the BCL-2 proteins, and the similarity to the protein transport channel formed by diphtheria toxin [38], it is possible that the anti-apoptotic BCL-2 proteins act as 'chauffeurs' by assisting in the removal of misfolded/denatured proteins, thereby limiting the ability of such proteins to induce hsp70 production and apoptosis.

The present study demonstrates the ability of Bcl-x_L overexpression to delay, but not ultimately protect against, MG132-induced apoptosis. These data are consistent with studies that reported that Bcl-2 overexpression is not able to overcome the apoptotic effects of proteasome inhibition [4,32]. However, the delay afforded by Bcl-x_L overexpression does suggest that it is having some effect on the abnormalities induced by proteasome inhibition, although it is also possible that its protective effects occur further downstream.

The inability of L-canavanine-stimulated increases in hsp70 to protect against MG132 was somewhat surprising, since the induction of hsp70 following proteasome inhibition confers thermotolerance against high temperatures [39]. Moreover, a number of other studies have reported the ability of pre-induction of heat-shock proteins to protect against a subsequent stress, including hypoxia, ischaemia, alcohol, H_2O_2 and heavy metals [39–41]. Taken together, this suggests that heat-shock proteins are involved in monitoring the build-up of misfolded/damaged proteins that could otherwise be toxic, and that elevated levels of hsp70 prior to subjection to stress can mitigate the pejorative effects of accumulating aberrant proteins.

Three potential mechanisms can be envisioned to explain the L-canavanine results. (i) While L-canavanine leads to the accumulation of analogue-substituted proteins, i.e. abnormal proteins, that are believed to be responsible for the accumulation of hsp70, it could be that newly synthesized hsp70 itself is aberrant or dysfunctional, owing to the incorporation of L-canavanine. (ii) Although hsp70 is induced 8 h after L-canavanine treatment (a time when no apoptosis is observed), it is possible that these cells are primed to undergo apoptosis due to the presence of misfolded/aberrant proteins; a condition exacerbated by MG132 treatment. (iii) Finally, the degree of increase in hsp70 levels observed after L-canavanine might not have been sufficient to offer any protection from MG132-induced apoptosis.

On the basis of previous reports, one mechanism to explain hsp70's involvement in MG132-induced apoptosis could involve this protein's ability to block SAPK/JNK activation. Meriin et al. [42] reported that treatment of U937 lymphoid cells with MG132 stimulated a steady increase in JNK activity, which correlated with an increase in apoptosis, and inhibition of JNK activity reduced MG132-induced apoptosis. Moreover, hsp70 induction stimulated by a brief treatment with MG132 was able to protect against other initiators of apoptosis by suppressing JNK activity. Gabai et al. [18] also reported that hsp70's cytoprotective capacity involved its ability to prevent SAPK/JNK activation. Overall, hsp70-mediated cytoprotection correlates with an inhibition of SAPK/JNK induced by various stimuli [42,43].

The role of caspases in proteasome-induced apoptosis remains unclear. An et al. [4] reported that the apoptosis-inducing effect of proteasome inhibition is completely inhibitable by YVAD-CMK, a tetrapeptide inhibitor of caspase activity. In contrast, both caspase-dependent and caspase-independent apoptosis regulated by the proteasome have been reported [44]. We found that a general caspase inhibitor was highly effective in blocking MG132-induced apoptosis, whereas a more specific caspase-3 inhibitor was only moderately effective, despite complete inhibition of Ac-DEVD-AMC cleavage by both compounds. This finding suggests that caspases other than caspase-3 are important in the effector phase of apoptosis induced by proteasome inhibition.

In summary, hsp70 antisense oligonucleotides blocked MG132-induced increases in this protein and significantly potentiated apoptosis. This clearly documents an important anti-apoptotic role for hsp70 in stressed cells. Bcl-x_L delayed, but did not prevent, proteasome-inhibitor-induced apoptosis, showing

that this protein has the same effect as Bcl-2. Overall, the interacting effects of hsp70 and the anti-apoptotic BCL-2 proteins suggest that they might be affecting some of the same pathways.

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