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Heat-shock pretreatment inhibits sorbitol-induced apoptosis in K562, U937 and HeLa cells

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The aim of this study was to determine whether heat-shock pretreatment exerted a protective effect against sorbitol-induced apoptotic cell death in K562, U937 and HeLa cell lines and whether such protection was associated with a decreased cytochrome c release from mitochondria and a decreased activation of caspase-9 and -3. Following heat-shock pretreatment ($42 \pm 0.3^\circ\text{C}$ for 1 hr), these cell lines were exposed to sorbitol for 1 hr. Apoptosis was evaluated by DNA fragmentation, whereas caspase-9, -3 activation, cytochrome c release and heat-shock protein70 (HSP70) were assayed by Western Blot. Sorbitol exposure-induced apoptosis in these different cell lines with a marked activation of caspase-9 and caspase-3, whereas heat-shock pretreatment before sorbitol exposure, induced expression of HSP70 and inhibited sorbitol-mediated cytochrome c release and subsequent activation of caspase-9 and caspase-3. Similarly, overexpression of HSP70 in the three cell lines studied prevented caspase-9 cleavage and activation as well as cell death. Furthermore, we showed that the mRNA expression of iNOS decreased during both the heat-shock treatment and heat-shock pretreatment before sorbitol exposure. By contrast, the expression of Cu-Zn superoxide dismutase (SOD) and Mn-SOD proteins increased during heat-shock pretreatment before sorbitol exposure. We conclude that, heat-shock pretreatment protects different cell lines against sorbitol-induced apoptosis through a mechanism that is likely to involve SOD family members.

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Key words: heat shock; HSP70; ROS; sorbitol; Cu-Zn-SOD; Mn-SOD

In multicellular organisms, apoptosis or programmed cell death is achieved by at least 2 independent pathways that are initiated and executed by distinct caspases, a class of cysteine proteases which plays a role in the dismantling of critical cellular components. For instance, several members of the tumor necrosis factor receptor family, such as Fas and tumor necrosis factor receptor-1, recruit pro-caspase-8 to their cytosolic domains upon ligation, to activate caspase-8, which subsequently activates the distal caspases-3, -6 and -7.¹ Another pathway of caspase activation involves mitochondria from which cytochrome c is released into the cytosol. Once released, cytochrome c binds with apoptosis protease activating factor-1 (Apaf-1) and activates caspase-9 in an ATP/dATP-dependent manner.^{2,3}

During the past decade, several proteins that promote tumorigenesis by inhibiting apoptosis have been identified in primary tumors including antiapoptotic members of the bcl-2 protein family, heat-shock proteins and members of the inhibitor of apoptosis protein family.⁴ Heat-shock proteins are highly conserved proteins known to protect cells from adverse environmental, physical and chemical stresses through their ability of preventing protein aggregation and promote the refolding of denatured proteins.⁵ The ability of such proteins to prevent apoptosis induced by several anticancer drugs also explains how these proteins could limit the efficacy of cancer therapy.^{6,7} Indirect experimental evidence and clinical-pathological studies indicate that HSP70 is the major stress inducible, cancer-associated, antiapoptotic protein.⁴ Increased expression of HSP70 has been reported in high grade malignant breast and endometrial

tumors, osteosarcoma and renal cell tumors.⁶ Understanding the molecular mechanisms of action of these proteins is necessary to initiate novel modes of rationally and selectively manipulating the sensitivity of cancer cells to therapy.⁴

Recently, we have shown that sorbitol is able to induce apoptosis efficiently and rapidly when provided at high concentrations in human K562 cells, and the death process is strictly related to a massive production of reactive radical species.^{8,9} In response to oxidant stress, however, cells can initiate protective responses to limit damage and ensure survival. Cellular protection in response to Reactive Oxygen Species (ROS) includes expression of antioxidant enzymes. Superoxide dismutase (SOD) is an antioxidant enzyme involved in the defense systems against ROS. There are 3 known SODs, including mitochondrial manganese SOD (Mn-SOD), intracellular copper, zinc SOD (Cu,Zn-SOD), which localizes into the cytoplasm and nucleus, and extracellular Cu,Zn-SOD. These SODs catalyze dismutation of 2 superoxide anions ($\text{O}_2^{\cdot-}$) into hydrogen peroxide, which is then catalyzed to innocuous O_2 and H_2O by glutathione peroxidase and catalase. Excessive ROSs provoke untoward events such as DNA damage, peroxidation of the lipid membrane and protein.

We report that heat-shock pretreatment increases the resistance of 3 different cell lines to sorbitol-induced apoptosis by inhibiting caspases activation and iNOS expression. Furthermore, some members of SOD family are likely to be involved in this process.

Material and methods

Cell culture and conditions

The chronic myelogenous K562 leukemia cells, the promyelocytic human leukemia U937 cells and the cervical carcinoma HeLa cells were obtained from American Type Culture Collection (ATCC). K562 and U937 cells were cultured in growth medium RPMI 1640, the HeLa in growth medium Dulbecco modified Eagle medium and all cell lines supplemented with 10% heat-inactivated fetal bovine serum at 37°C in the presence of 5% CO_2 under a humidified atmosphere. All cultures were maintained in an incubator at 37°C (in 5% $\text{CO}_2/95\%$ air).

Heat-shock treatment

The 3 cell lines underwent different treatments: (i) hyperthermia of $42 \pm 0.3^\circ\text{C}$ for 1 hr by immersing the flasks in circulating

Additional supporting information may be found in the online version of this article.

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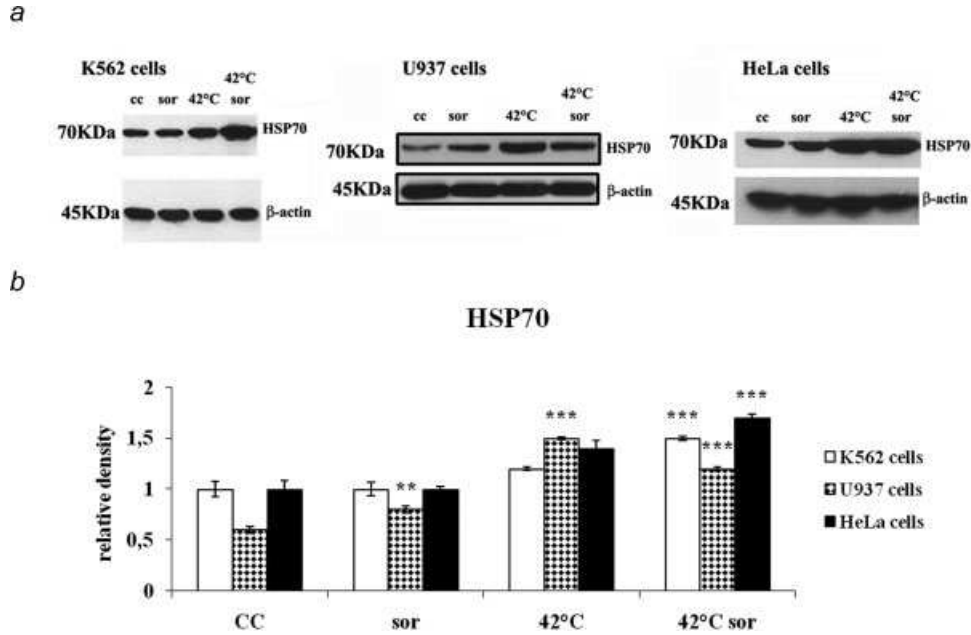


FIGURE 1 – Effects of heat-shock treatment, sorbitol treatment and heat-shock pretreatment before sorbitol incubation in K562, U937 and HeLa cell lines on HSP70 protein expression. (a) Whole-cell lysates were prepared from untreated cell lines (lanes cc), and cell lines treated as indicated. HSP70 was detected by Western blot using anti-HSP70 antibodies. β-actin was used as a control for protein loading. The molecular weight (in KDa) of protein size standards is shown on the left hand side. (b) Densitometry analysis of the blots shown in (a). Results are expressed as the mean ± SE of 3 independent experiments performed in triplicate. Significant differences between the cell lines treated in different way and untreated cell lines are indicated by probability *p*. **p* < 0.05, ***p* < 0.01 and ****p* < 0.001; ANOVA with Tukey’s post-test.

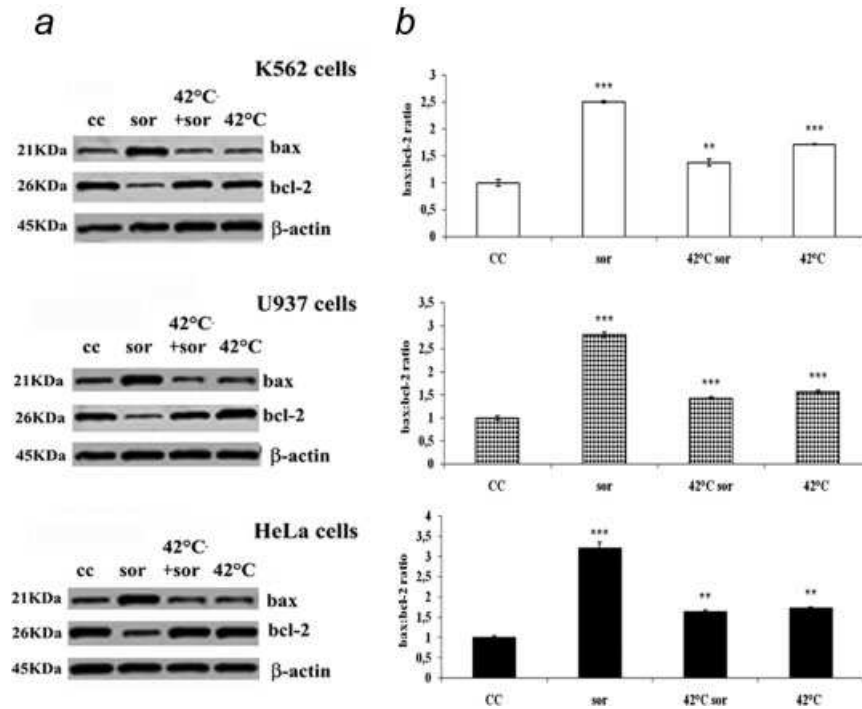


FIGURE 2 – Effects of heat-shock treatment, sorbitol treatment and heat-shock pretreatment before sorbitol incubation in K562, U937 and HeLa cell lines on bcl-2 family proteins. (a) Analysis of bax and bcl-2 proteins expression. Whole-cell lysates were prepared from untreated cell lines (lanes cc) and cell lines treated as indicated. Western blot analysis was performed with an antibody, which specifically recognized bax, bcl-2 and β-actin. β-actin was used as loading control. The molecular weight (in KDa) of protein size standards is shown on the left hand side. Blots are representative of at least three separate experiments. (b) Bax:bcl-2 ratio in untreated cell lines (lane cc) and treated cell lines were obtained by densitometry analysis of the blots shown in (a). Significant differences among the value of the sorbitol treatment, heat-shock treatment and heat-shock pretreatment before sorbitol incubation are indicated by probability *p*. **p* < 0.05, ***p* < 0.01 and ****p* < 0.001; ANOVA with Tukey’s post-test.

water bath; (ii) hyperthermia of $42 \pm 0.3^\circ\text{C}$ for 1 hr by immersing the flasks in circulating water bath followed by treatment with 1 M sorbitol for 1 hr; (iii) addition of 1 M sorbitol for 1 hr. Immediately after the different treatments, all cell lines were transferred to a 37°C incubator for 3 hr. As a control, cells were cultured under normal conditions without hyperthermia. After recovery, all cell lines were used for the following *in vitro* experiments.

Cell viability

After exposure to different treatment, cell viability was assessed by propidium iodide (PI) exclusion. Cells (0.5×10^6) were resuspended in phosphate-buffered saline (PBS) and 50 $\mu\text{g/ml}$ PI before flow cytometric analysis (FACScan; Becton Dickinson, Oxford, UK).

DNA fragmentation assay

Cells were washed twice with PBS and lysed by addition of a hypotonic solution (1% NP-40 in 20 mM EDTA, 50 mM Tris-HCl pH 7.5). The supernatant was collected and processed as previously reported.¹⁰

Protein extraction and western blot analysis

Cells were lysed in 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 $\mu\text{g/ml}$ aprotinin, 0.5 mM sodium orthovanadate and 20 mM sodium pyrophosphate. Lysates were centrifuged at $14,000 \times \text{rpm}$ for 10 min. Protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, CA). Equivalent amounts of protein were loaded and electrophoresed on SDS-polyacrylamide gels. Subsequently, proteins were transferred to nitrocellulose membranes (Immobilon, Millipore Corp., Bedford, MA). After blocking with Tris-buffered saline BSA (25 mM Tris (pH7.4), 200 mM NaCl and 5% BSA), the membrane was incubated with the following primary antibodies: anti-HSP70 MAb, anti-bax MAb, anti-bcl-2 MAb, anti-caspase-9 MAb, anti-procaspase-3 MAb, anti-caspase-3 active (cleaved) PAb (Millipore Corp.), fragment anti PARP MAb, anti-Mn-SOD MAb, anti-Cu-Zn-SOD MAb- (Santa Cruz Biotechnology) and anti- β -actin MAb (Sigma). Membranes were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody, and the reaction was detected by an enhanced chemiluminescence system (Amersham Life Science, Buckinghamshire, UK). The relative amount of protein expression was quantified using Gel-Doc phosphorimager and Quantity One software (Bio-Rad) and normalized by the intensity of β -actin.

Caspase-3 activity assay

Cells (1×10^6) were incubated for 1 h at 37°C with sorbitol 1 M. Subsequently, cells were incubated for 2 hr in fresh medium without sorbitol, then washed in PBS, pelleted in a microcentrifuge, and frozen at -80°C . Cells were thawed and resuspended in 50 μl PBS. The appropriate peptide substrate (DEVD-7-amido-4-methylcoumarin AMC) was added according to the manufacturer's instructions (Calbiochem). Fluorescence was measured in a FACSCalibur Becton Dickinson.

Cytosolic protein extraction and cytochrome c immunoblot

Isolation of mitochondria and cytosolic fraction and cytochrome c immunoblot were carried out using a modified protocol from Kluck *et al.*¹¹ Cells were treated and then lysed in lysis buffer (1 mM CaCl_2 , 1 mM MgCl_2 , 1% NP-40, 1 $\mu\text{g/ml}$ leupeptin, 1 $\mu\text{g/ml}$ aprotinin, 1 μM PMSF, and 100 μM NaVO_4). Samples were then incubated on ice for 20 min and centrifuged at $14,000 \text{ rpm}$ for 15 min. The supernatants were collected, and protein concentration was determined by the Bradford assay. About 80 μg of protein were loaded in a 10% SDS-PAGE gel and electrophoresed. The membrane was incubated with the primary anticytochrome c polyclonal antibody (Santa Cruz) followed by incubation with a horseradish peroxidase conjugated with anti-rabbit secondary antibody.

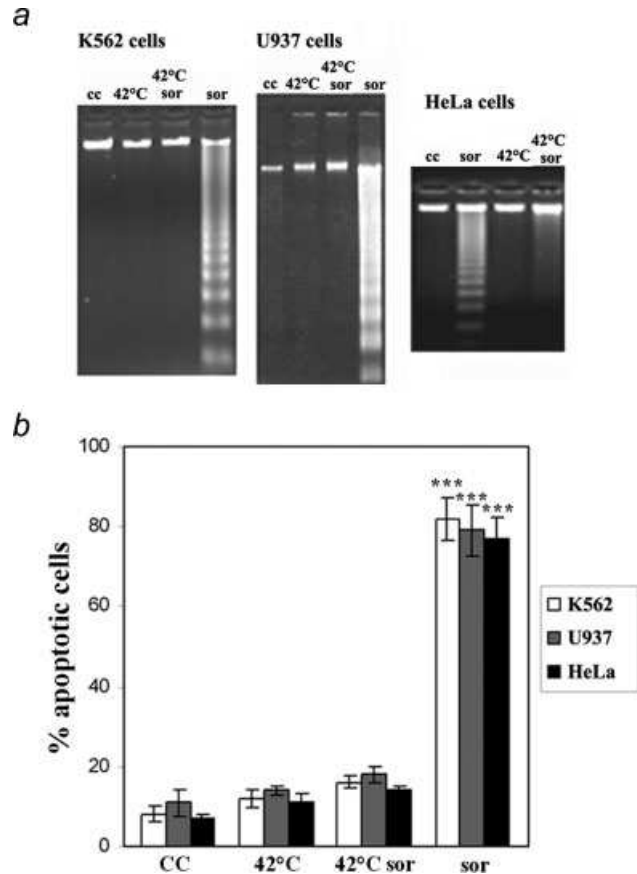


FIGURE 3 – Effects of heat-shock treatment, sorbitol treatment and heat-shock pretreatment before sorbitol incubation in K562 and U937 and HeLa cell lines on DNA Fragmentation. (a) DNAs were extracted from untreated cell lines (lanes cc) and treated cell lines. Agarose gels are representative of at least 3 separate experiments. (b) Percentage of the cell death quantified by measuring PI-uptake by flow cytometry. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$; ANOVA with Tukey's post-test.

The reaction was detected with an enhanced chemiluminescence system. The relative amount of protein expression was quantified using a Gel-Doc phosphorimager and Quantity One software (Bio-Rad) and normalized by the intensity of β -actin.

HSP70 transient transfection assay

K562, U937 and HeLa cells were transfected with an empty pBK-CMV vector or with a pBK-CMV vector plus 1.6 kb hsp70 cDNA using Lipofectamine liposomal transfection reagent (Boehringer Mannheim). Briefly, the pBK-CMV-HSP70 plasmid was diluted to a concentration of 0.1 $\mu\text{g}/\mu\text{l}$ in 20 mM Hepes buffer (pH 7.5). The DNA solution was added to diluted DOTAP liposomal transfection reagent (Boehringer) containing 240 μl of DOTAP and 720 μl of 20 mM Hepes buffer (pH 7.5). The transfection mixture was incubated at room temperature for 15 min, mixed with 6.5 ml of culture medium containing 5% fetal calf serum, penicillin and streptomycin for all cell lines. The day after, cells were washed with PBS, collected and the expression of HSP70 assayed by western blot.

RNA isolation and northern blot

Total RNAs from control or treated cells were isolated using Tri Reagent (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). Aliquots of RNA were electrophoresed on 1% agarose formaldehyde gels and subsequently blotted onto nylon membranes (Hybond N, Amersham, Braunschweig, Germany). The membrane was then UV crosslinked, and hybridized to ^{32}P -labeled probe.

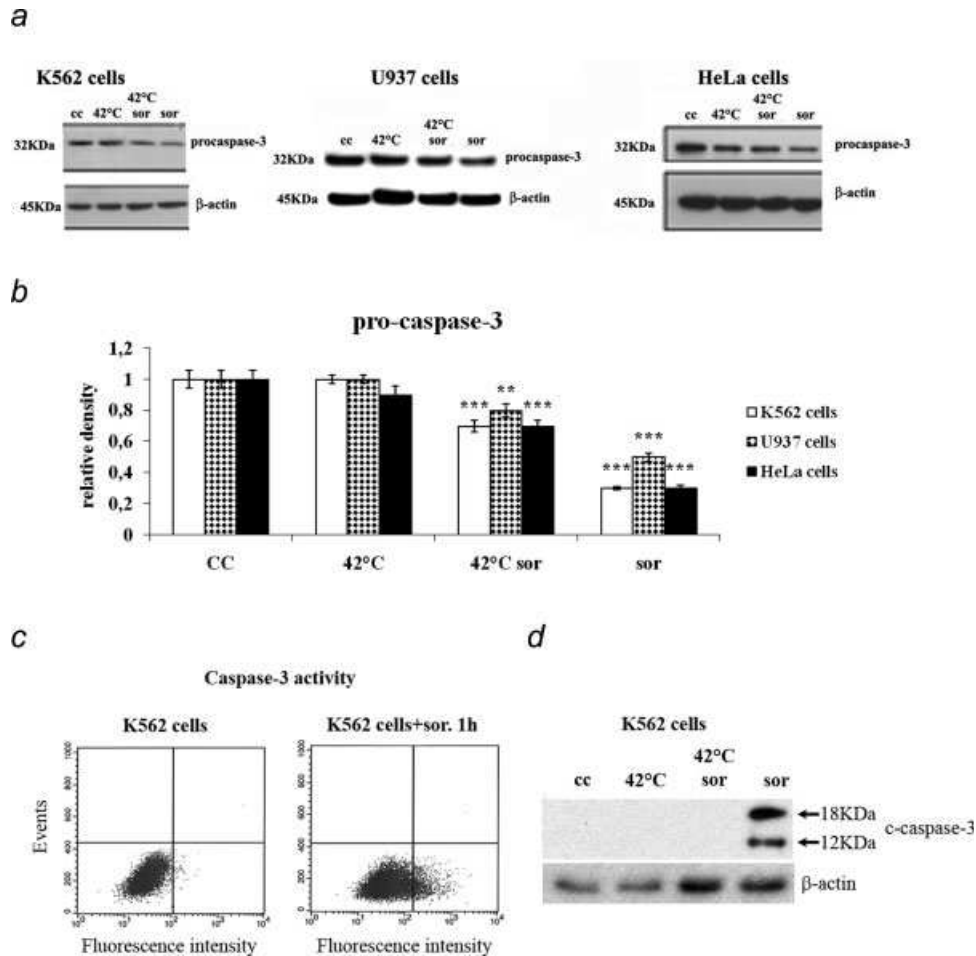


FIGURE 4 – Effects of heat-shock treatment, sorbitol treatment and heat-shock pretreatment before sorbitol incubation in K562 and U937 and HeLa cell lines on the caspase-3 activation (a) Whole-cell lysates were prepared from untreated cell lines (lanes cc) and treated all cell lines. Cellular extracts were analyzed by Western blot using anti-procaspase-3 antibodies. β -actin was used as a control for protein loading. The molecular weight (in KDa) of protein size standards is shown on the left hand side. Blots are representative at least 3 separate experiments. (b) Densitometry analysis of the blots shown in (a). Results are expressed as the mean \pm SE of 3 independent experiments performed in triplicate. Significant differences between the cell lines treated in different way and untreated cell lines are indicated by probability p . * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$; ANOVA with Tukey's post-test. (c) Caspase-3 activity was measured in K562 cells either left untreated or treated with sorbitol for 1 hr using a fluorescent substrate as described under Material and Methods. Change in fluorescence intensity was measured with a FACSCalibur Becton Dickinson flow cytometer. (d) K562 cells were left either untreated or treated as indicated. Generation of cleaved active forms of caspase-3 was measured by western blot assay using an antibody specifically recognizing caspase-3 cleaved fragments. β -actin was used as loading control.

Statistical analysis

Data are presented as mean \pm SEM. One-way ANOVA with Tukey's post-test was performed using GraphPad InStat Version 3.00 for Windows 95 (GraphPad Software, San Diego, CA).

Results

Heat-shock pretreatment prevents bax increase and bcl-2 decrease induced by sorbitol

The 3 cell lines studied were subjected to different treatments: (i) heat shock ($42 \pm 0.3^\circ\text{C}$ for 1 hr), (ii) heat shock ($42 \pm 0.3^\circ\text{C}$ for 1 hr) followed by incubation with 1 M sorbitol for 1 hr, (iii) incubation with 1 M sorbitol alone. After the indicated treatments, cells were examined by Western blotting. Figure 1 shows a significant ($p < 0.001$) higher expression level of HSP70 in the heat shocked cell lines in the presence or absence of sorbitol when compared with control cells or cells treated with sorbitol alone (Figs. 1a and 1b). Furthermore, sorbitol treatment increased expression of the bax protein while decreasing expression of bcl-2 protein. By contrast, when sorbitol incubation was preceded by heat-shock treatment, we did not detect

any change in the cellular content of bax and bcl-2 in the 3 cell lines examined (Fig. 2a). Since the antagonism between bax and bcl-2 is crucial for the apoptotic process regulation, we have measured the intensity of the bands of bax and bcl-2 from Figure 2a. Figure 2b shows that bax:bcl-2 ratio was statistically significant ($p < 0.001$) in all cell lines treated with sorbitol whereas, heat-shock pretreatment reduced this ratio as compared with that of control cells.

Heat-shock pretreatment protects the three cell lines from sorbitol-induced DNA degradation

During apoptosis endonucleases degrade the DNA structure, producing DNA fragments (folds of 180–200 bp) which are released from nuclei into cytosol. Electrophoresis of genomic DNA from apoptotic cells shows a characteristic laddering pattern. To confirm that sorbitol-induced apoptosis in K562, U937 and HeLa cells, we have examined DNA laddering formation by agarose gel electrophoresis. When cells were exposed to 1 M sorbitol for 1 hr, a prominent DNA laddering was observed (Fig. 3a). Importantly, heat-shock pretreatment protected K562, U937 and HeLa cells from sorbitol-induced apoptosis. In fact, Figure 3a shows that DNA

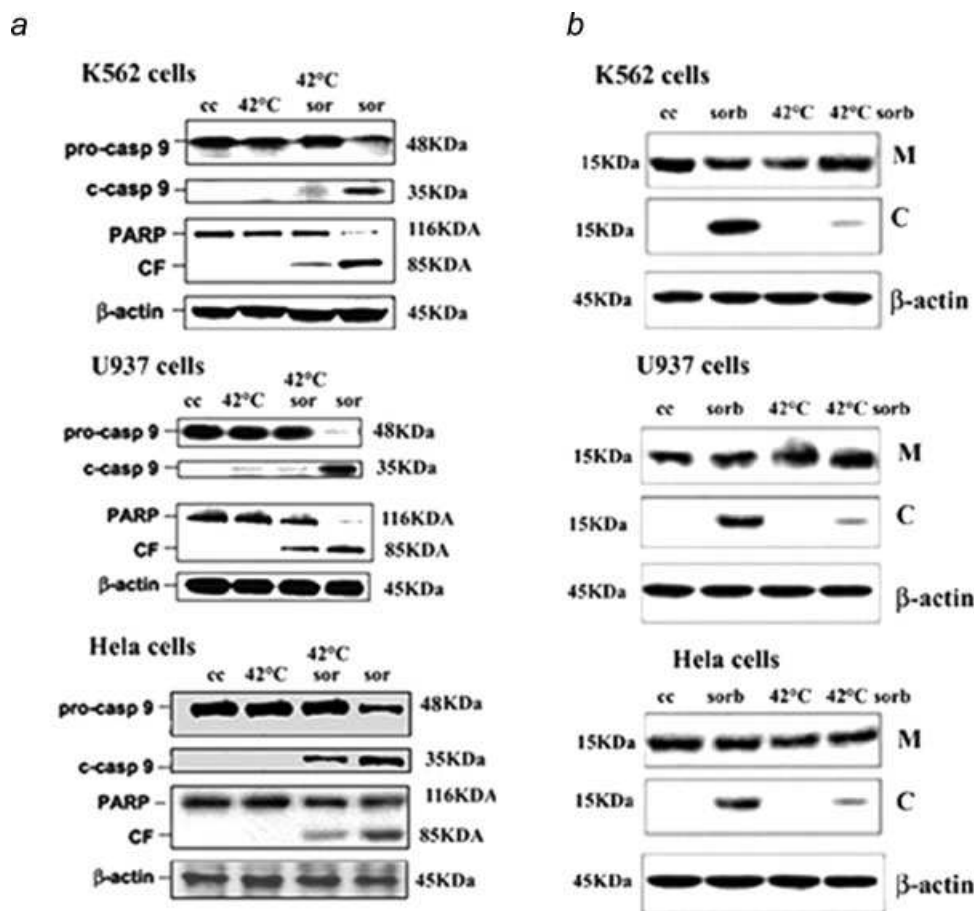


FIGURE 5 – Effects of heat-shock treatment, sorbitol treatment and heat-shock pretreatment before sorbitol incubation in K562 and U937 and HeLa cell lines on caspase-9 activation, PARP cleavage and cytochrome *c* release. (a) Whole-cell lysates were prepared from untreated (lanes cc) and treated cell lines. Cellular extracts were analyzed by Western blot using caspase-9 and PARP specific antibodies. β -actin was used as a loading control. The molecular weight (in KDa) of protein size standards is shown on the right hand side. Blots are representative at least 3 separate experiments. (CF = cleaved fragment), (c-caspase-9 = cleaved caspase-9). (b) Mitochondrial (M) and cytosolic (C) fraction were prepared from untreated (lanes cc) and treated cell lines as described under Material and Methods. Equal amounts of protein from each sample were subjected to Western blot analysis, and probed for cytochrome *c*. β -actin was used as a loading control. The molecular weight (in KDa) of protein size standards is shown on the left hand side. Blots are representative at least 3 separate experiments. (M = mitochondria). (C = cytosol).

degradation was decreased significantly when cell lines were pretreated with heat shock before sorbitol exposure.

Similar results were obtained by flow cytometry analysis after PI staining. Figure 3b shows that heat-shock pretreatment reduced the number of apoptotic cells to 20% from 80% of sorbitol alone.

We next examined whether heat-shock pretreatment had a protective effect after prolonging the sorbitol treatment up to 24 and 48 hr. Supporting information Figure 1 shows that 24 and 48 hr treatment with sorbitol resulted in the death of more than 90% of the cells for the 3 cell lines examined. Importantly, cell killing by sorbitol was still substantially reduced by the heat-shock pretreatment. In fact, after 24 and 48 hr in the K562 and HeLa cells, the percentage of cell killing was reduced to 30 and 20%, respectively (Supporting information Fig. 1). In the U937 cell line the percentage of cell death was reduced to 40% (Supporting Information Fig. 1).

Heat-shock pretreatment suppresses the activation of procaspase -3 and proteolytic caspase-9

We next examined whether the morphological features of apoptosis were accompanied by caspase-3 and caspase-9 activation. Caspase-3 is a critical effector caspase of the apoptotic process. Caspase-9 is an initiator of caspase-3 in the mitochondria-dependent pathway. Western blot analysis of whole-cell lysates, obtained

from the cell lines treated with 1 M sorbitol for 1 hr, revealed a clear ($p < 0.001$) decrease of procaspase-3 (Figs. 4a and 4b). By contrast, heat-shock pretreatment before sorbitol exposure resulted in a slight decrease of procaspase-3 (Fig. 4a). Activation of caspase-3 was more accurately measured by flow cytometry using a fluorescent substrate and western blot using an antibody that specifically recognizes the cleaved active form of caspase-3. Figures 4c and 4d show that sorbitol treatment induced a rapid activation of caspase-3 due to the generation of cleaved active forms. Similarly, exposure to 1 M sorbitol of the 3 cell lines, induced a proteolytic cleavage of caspase-9, as revealed by the appearance of the characteristic fragment on the Western immunoblot (Fig. 5a). Such proteolytic fragments were decreased significantly in lysates from the same cell lines pretreated with heat shock before being exposed to sorbitol (Fig. 5a). Furthermore, PARP cleavage was decreased significantly in lysates from cells pretreated with heat shock and subsequently incubated with sorbitol (Fig. 5a).

Effect of heat-shock pretreatment on cytochrome c release in K562, U937 and HeLa cell lines treated with sorbitol

Cytochrome *c* acts as an important molecule at the early stage of the apoptotic pathway. Its release from mitochondria leads to caspase-9 activation, which then converts procaspase-3 into its active form, resulting in the executionary phase of apoptosis. Western blot analysis on mitochondrial and cytosolic fractions

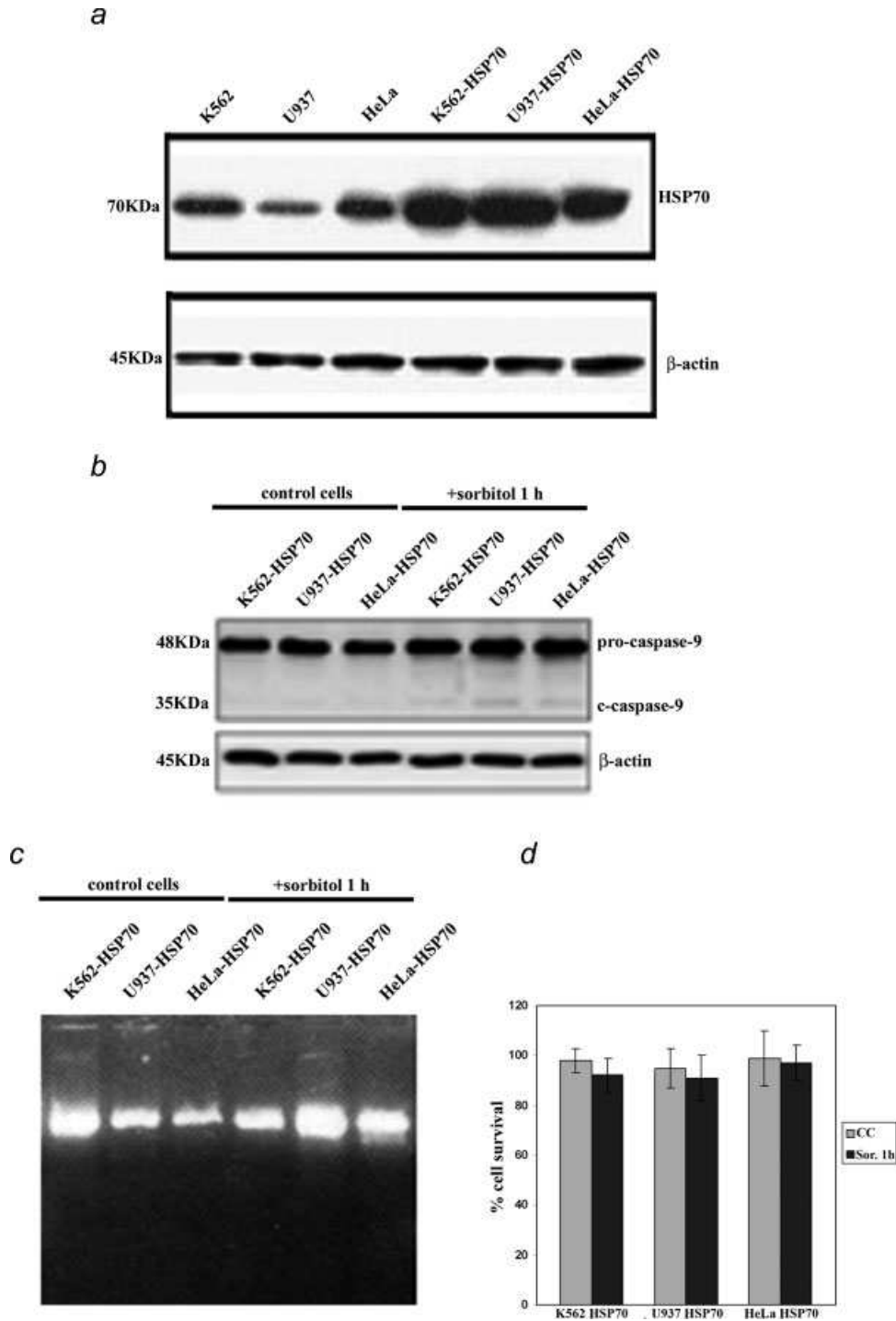


FIGURE 6 – Effects of HSP70 overexpression on caspase-9 cleavage, DNA fragmentation and cell survival. (a) K562, U937 and HeLa cells were transiently transfected with a mock vector or with a pBK-CMV- hsp70 vector. HSP70 overexpression was measured by western blot. (b) The 3 cell lines were left either untreated or treated with sorbitol for 1 hr. Generation of a cleaved fragment of caspase-9 was assayed by western blot. (c-caspase-9 = cleaved caspase-9). (c) DNAs were extracted from untreated and sorbitol-treated cell lines. Agarose gels are representative of at least three separate experiments. (d) Percentage of the cell survival quantified by measuring PI-uptake by flow cytometry.

were performed to address whether heat-shock pretreatment of all cell lines was able to block sorbitol-induced cytochrome c release. Figure 5b shows that cytochrome c increased in the cytosolic fraction after treatment with sorbitol. Importantly, heat-shock pretreatment before sorbitol exposure, significantly reduced the content of cytochrome c detected in the cytosolic extracts from the cell lines examined (Fig. 5b). Therefore, HSP70 overexpression could block

apoptosis at some point upstream of caspase-3 activation, by affecting cytochrome c release.

Heat-shock protein overexpression suppresses activation of caspase-9 and prevents DNA fragmentation and cell death

Figure 6a shows that a transient transfection of the 3 cell lines examined with a mammalian expression vector resulted in an

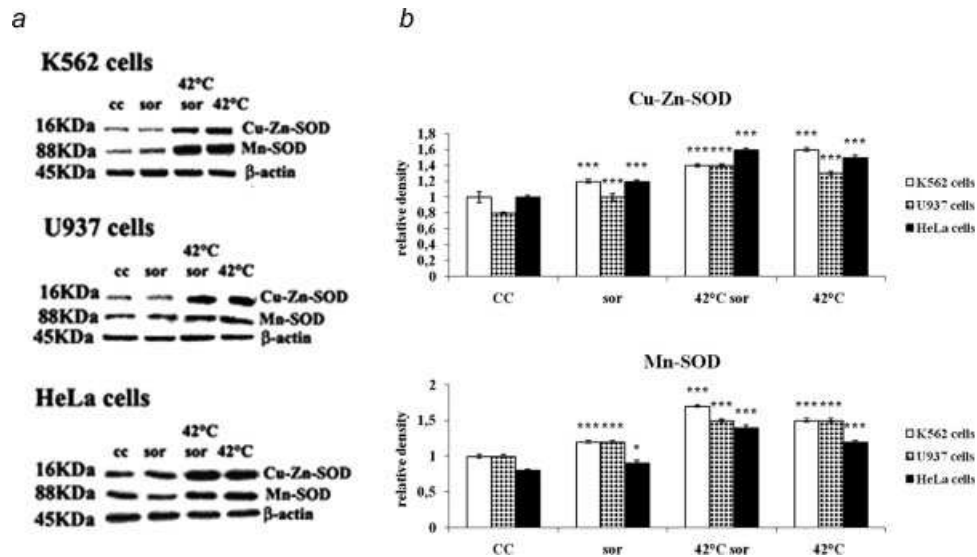


FIGURE 7 – Effects of heat-shock treatment, sorbitol treatment and heat-shock pretreatment before sorbitol incubation of sorbitol in K562, U937 and HeLa cell lines on Mn- and Cu-Zn-SOD expression. (a) Whole-cell lysates were prepared from untreated (lanes cc) and treated cell lines as indicated. Cell lysates were collected and equal amounts of protein from each sample were subjected to Western blot analysis and probed for Mn- and Cu-Zn-SOD. β-actin was used as a control for protein loading. The molecular weight (in KDa) of protein size standards is shown on the left hand side. Blots are representative at least 3 separate experiments. (b) Densitometry analysis of the blots shown in (a). Results are expressed as the mean ± SE of 3 independent experiments performed in triplicate. Significant differences between the cell lines treated in different way and untreated cell lines are indicated by probability *p*. **p* < 0.05, ***p* < 0.01 and ****p* < 0.001; ANOVA with Tukey’s post-test.

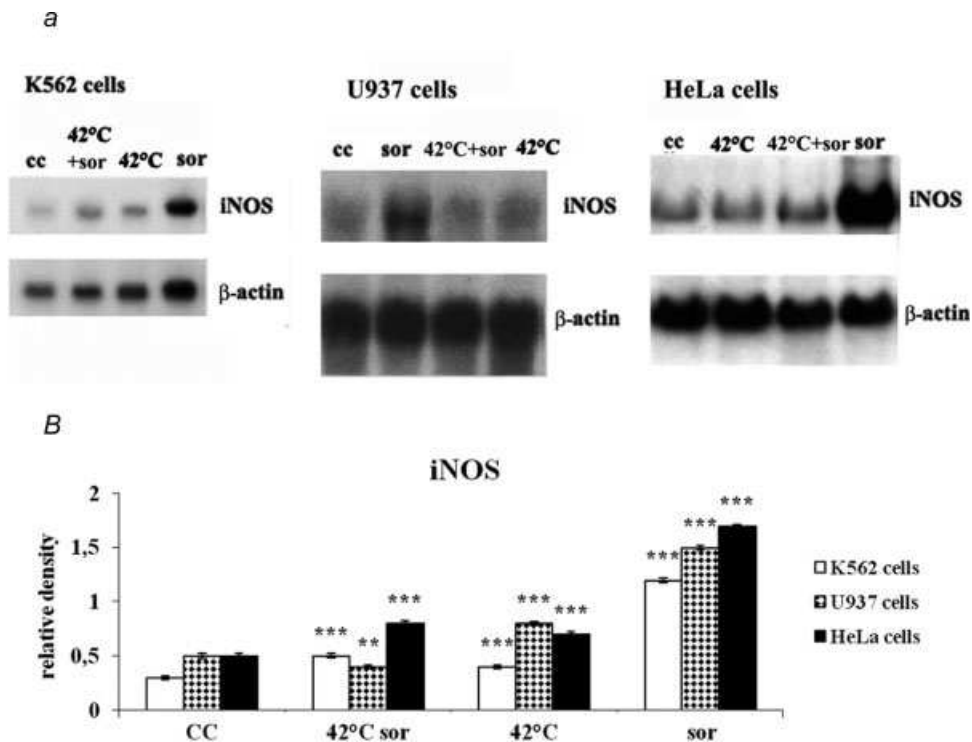


FIGURE 8 – Effects of heat-shock treatment, sorbitol treatment and heat-shock pretreatment before sorbitol incubation in K562, U937 and HeLa cell lines on iNOS mRNA expression. (a) RNAs extracted from untreated (lanes cc) and treated cell lines as indicated, were electrophoresed and hybridized with a labeled probe for iNOS as described under Materials and Methods. β-actin was used as loading control. Blots are representative of at least 3 separate experiments. (b) Densitometry analysis of the blots shown in (a). Results are expressed as the mean ± SE of 3 independent experiments performed in triplicate. Significant differences between the cell lines treated in different way and untreated cell lines are indicated by probability *p*. **p* < 0.05, ***p* < 0.01 and ****p* < 0.001; ANOVA with Tukey’s post-test.

overexpression of the HSP70 protein. When the HSP70 overexpressing cells were treated with sorbitol for 1 hr, we did not observe any cleavage of caspase-9 (Fig. 6b). HSP70 overex-

pression and inhibition of caspase-9 cleavage was followed by the absence of a detectable DNA fragmentation in the 3 cell lines (Fig. 6c). Furthermore, overexpression of HSP70 prevented

sorbitol toxicity and maintained the viability of treated cells to a percentages similar to that of controls (Fig. 6d).

Effect of heat-shock pretreatment on Mn-SOD and Cu-Zn-SOD expression in K562 and U937 and HeLa cell lines treated with sorbitol

The impact of the heat-shock pretreatment before sorbitol exposure on Mn-SOD and Cu-Zn-SOD expression in the cell lines used is shown in Figure 7a and 7b. Western blot analysis of whole-cell lysates, obtained from the cell lines treated with 1 M sorbitol, revealed a significant decrease of Mn-SOD and Cu-Zn-SOD expression after 1 hr. By contrast, western blot analysis showed that heat-shock treatment alone as well as heat-shock pretreatment followed by sorbitol exposure were able to significantly increase the expression of both Mn- and Cu-Zn SOD (Figs. 7a and 7b).

Effect of heat-shock pretreatment on iNOS mRNA expression in K562 and U937 and HeLa cell lines treated with sorbitol

In a previous study, we have demonstrated the increase in the content of inducible NOS (iNOS) mRNA at 30 min from sorbitol treatment using Northern blot and Western blot.⁸ The upregulation of iNOS expression was confirmed by the parallel increase in iNOS protein content as determined by Western blot analysis.⁸ Importantly, we also showed that pretreatment of K562 cells with N^o-nitro-L-arginine methyl ester and L-N⁶- (L-iminoethyl) lysine hydrochloride, 2 specific NOS inhibitors, prevented DNA fragmentation during sorbitol treatment.⁸ As shown in Figures 8a and 8b, treating the cell lines with sorbitol alone for 1 hr substantially increased the steady state levels of iNOS mRNA. By contrast, heat-shock pretreatment significantly attenuated sorbitol-mediated iNOS expression in the 3 cell lines used (Figs. 8a and 8b). The housekeeping gene β -actin was not affected by the experimental conditions.

Discussion

HSPs are evolutionarily-conserved molecular chaperones that are essential for the proper folding and assembly of proteins. The proteins are structurally and functionally conserved from prokaryotes to mammals. HSP70 provides protection from elevated temperature and contributes to thermotolerance. This HSP70 ability to protect cells has been shown recently to be a consequence of an apoptosis inhibition.¹²⁻¹⁶

The protective capacity of HSPs is classically defined by their ability to confer a state of "thermotolerance," wherein exposure to a mild heat shock renders cells transiently resistant to subsequent, more damaging, high temperatures.^{17,18} Furthermore, the acquisition of thermal tolerance, either by cells exposure to a sublethal temperature or by HSPs overexpression, specifically HSP70 and HSP27, is able to confer a more general resistance, by inhibiting apoptosis in response to a variety of stimuli.^{19,20} These stimuli include increased temperature,²¹ chemotherapeutics,²² ultraviolet radiation²³ and tumor necrosis factor.²⁴

We have found that heat-shock-induced thermotolerance of 3 different cell lines (K562, U937 and HeLa) induced high levels of HSP70 and protects these cells from stress-induced apoptosis by sorbitol.

Heat-shock pretreatment induces the HSP70 synthesis that confers a protective effect against a wide range of cellular stresses. HSPs help maintaining the metabolic and structural integrity of the

cell as a protective response to external stresses. However, the molecular mechanism by which HSPs inhibit apoptosis has not been well explored. Recent progress in HSPs studies and apoptosis have revealed that HSPs function at key regulatory points in the apoptosis control, directly interacting with different apoptotic proteins. Furthermore, there is very little information on the effects of hyperthermia on the activity of proapoptotic bcl-2 family members, and how heat-shock proteins might regulate their activities. A recent report suggests that inhibition of bax translocation to mitochondria by HSP70 is the crucial factor in the cellular decision to either trigger or suppress apoptosis.²⁵ We have focused on the pro- and anti-apoptotic bcl-2 family members because of their central role in regulating cytochrome c release in stressed cells. We have found that sorbitol treatment increased expression of the bax protein while decreasing the expression of bcl-2 protein. However, we did not detect any change in the cellular content of bax and bcl-2 when the cell lines were subjected to heat-shock treatment or heat-shock pretreatment before sorbitol exposure.

In addition, ROS can induce apoptosis by evoking mitochondrial permeability transition (PT) or by directly activating caspase-3-like proteases. Furthermore, the activated caspases have been also shown to target and open the mitochondrial PT pore. Initiation of PT reduces mitochondrial transmembrane potential $\Delta\Psi_m$ is an irreversible step of programmed cell death,²⁶ resulting in release of cytochrome c and caspase zymogens from the mitochondrial inner-membrane space to the cytosol.²⁷ In our previous reports,^{8,9} we have demonstrated that ROS production and $\Delta\Psi_m$ reduction are involved in sorbitol-induced apoptosis. Thus, in this study, we have showed by Western blot, that expression of Mn-SOD and Cu-Zn-SOD proteins decreased during treatment with sorbitol in all cell lines. By contrast, the expression of the same proteins increased when the 3 cell lines were subjected to heat-shock pretreatment and then incubated with sorbitol.

We have also found that, similarly to the heat-shock pretreatment, overexpression of HSP70 protein is able to protect the 3 different cell lines used from sorbitol-induced apoptosis. However, it remains unclear whether it is the newly expressed HSPs or some other aspects of the heat-shock pretreatment, which regulates apoptosis upstream of mitochondria in this model of sorbitol-induced apoptosis. From these results, we have surmised that Hsp70 could directly enhance and inhibit the activity of key molecules acting at the execution phase of apoptosis to prevent cell death. It remains to be determined whether HSP70 directly interacts with a component of the pathway leading from cytochrome c release to caspase-3 activation.

It has recently been suggested that other HSPs, HSP27, HSP60 and HSP90 are also important regulators of apoptosis.²⁸⁻³⁰ In addition to protective effects, in some situations various HSPs have been found to accelerate apoptotic processes.²⁸⁻³⁰ An inappropriate apoptotic mechanism is implicated in a number of human diseases, understanding the molecular mechanisms of action of these proteins could offer novel ways of treating apoptosis-related diseases. Several HSPs, especially HSP70, might be useful for the therapeutic treatment of cancer, autoimmune diseases and neurodegenerative disorders.

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