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Molecules and Cells

The Heat Shock Protein 27 (Hsp27) Operates Predominantly by Blocking the Mitochondrial-Independent/Extrinsic Pathway of Cellular Apoptosis

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Heat shock protein 27 (Hsp27) is a molecular chaperone protein which regulates cell apoptosis by interacting directly with the caspase activation components in the apoptotic pathways. With the assistance of the Tat protein transduction domain we directly delivered the Hsp27 into the myocardial cell line, H9c2 and demonstrate that this protein can reverse hypoxia-induced apoptosis of cells. In order to characterize the contribution of Hsp27 in blocking the two major apoptotic pathways operational within cells, we exposed H9c2 cells to staurosporine and cobalt chloride, agents that induce mitochondria-dependent (intrinsic) and -independent (extrinsic) pathways of apoptosis in cells respectively. The Tat-Hsp27 fusion protein showed a greater propensity to inhibit the effect induced by the cobalt chloride treatment. These data suggest that the Hsp27 predominantly exerts its protective effect by interfering with the components of the extrinsic pathway of apoptosis.

INTRODUCTION

Apoptosis or programmed cell death can be mediated by two major pathways, either involving the Fas protein (extrinsic) or the mitochondria (intrinsic) (Concannon et al., 2003; Gewies, 2003). Binding of the Fas receptor by its ligand Fas at the cell surface initiates the first pathway culminating in the activation of the caspase-8 protease and the downstream caspase cascade (Charette et al., 2000; Concannon et al., 2003; Sartorius et al., 2001). The intrinsic pathway is triggered due to cell injury by DNA damage, cell distress by procedures such as ischemia/reperfusion, or chemotherapeutic drugs (Ashkenazi, 2002), leading to the disruption of mitochondria releasing mediators like Smac/Diablo, apoptosis inducing factor (AIF) and cytochrome C into the cytoplasm and finally the activation of the caspase cascade (Bruey et al., 2000; Concannon et al., 2001; Li et al., 2009; Pandey et al., 2000; Paul et al., 2002; Slee et al., 1999).

Heat shock proteins (Hsp) are a family of proteins that inhibit aggregation and promote the refolding of denatured proteins. These molecular chaperones are induced upon exposure to environmental and physiological stresses (Ehrnsperger et al., 1997), reactive oxygen stress, heat, ischemia and bacterial endotoxins (Arya et al., 2007; Clark and Muchowski, 2000; Concannon et al., 2003; Delogu et al., 2002). The principal five conserved classes of heat-shock proteins are Hsp100, Hsp90, Hsp70, Hsp60, Hsp33, and the small heat-shock proteins (sHsps). Among the sHsps, the heat shock protein 27 (Hsp27), has strong anti-apoptotic properties and interacts with several members of the apoptotic signaling pathways (Garrido, 2002) consequently having protective effects in a variety of human apoptosis-related malignancies, including neuronal (Latchman, 2005) and vascular diseases (Delogu et al., 2002; Ferns et al., 2006). Recently it was also demonstrated that Hsp27 can effectively protect from ischemia/reperfusion induced heart failure in the rat model (Kwon et al., 2007). While several papers demonstrate a role for Hsp27 in either the intrinsic or extrinsic pathways, no paper has compared the effects side by side using a combination of chemical agents that are known to specifically interfere in the individual pathways.

In order to determine the anti-apoptotic pathway Hsp-27 affects, we delivered recombinant Hsp27 into apoptotic cells by employing a protein transduction domain (PTD), a useful tool for the direct delivery of proteins across cell membranes by a mechanism termed as 'protein transduction' (Derossi et al., 1994; Futaki et al., 2003; Prochiantz, 2000; Wadia and Dowdy, 2003; Zhao and Weissleder, 2004). The most common PTD, Tat (49-57) from HIV, has been demonstrated to be effective in directly transducing and delivering cargoes under both *in vitro* and *in vivo* conditions (Arakawa et al., 2007; Hotchkiss et al., 2006; Zhao and Weissleder, 2004). Using the Tat-peptide for the intra-cytoplasmic delivery of Hsp-27, we demonstrate the protective effect of Tat-Hsp-27 in preventing hypoxia-induced cellular apoptosis. For analyzing the role of Tat-Hsp27 in blocking apoptosis, we induced cell death with either cobalt chloride

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($CoCl_2$) or staurosporine. $CoCl_2$ has been reported as a hypoxia/ischemic-mimicking agent (Chandel et al., 1998; Jung and Kim, 2004; Jung et al., 2007; Zou et al., 2001) that activates the Fas-dependent pathway and stimulates reactive oxygen species (ROS) through a mitochondria-independent mechanism. On the other hand, it is widely known that staurosporine (STA) is a strong pro-apoptotic agent which induces the mitochondria-dependent apoptotic pathway in cells (Columbaro et al., 2001; Yue et al., 1998). We investigated the impact of Tat-Hsp27 mediated blockade on both apoptotic pathways by separately induced each condition.

MATERIALS AND METHODS

Expression and purification of fusion proteins

Tat-Hsp27 and Tat-GFP fusion constructs were generated by cloning an oligo corresponding to the 11 amino acid Tat sequence (TAT GGC AGG AAG AAG CGG AGA CAG CGA CGA CGA) along with the coding sequence of human Hsp27 or GFP in frame with the 6-His tag in the bacterial expression vector pRSET-A (Invitrogen). Hsp-27 coding sequence was PCR amplified from a full length human Hsp27 cDNA using the oligo primers 5′-CACGAGGAGCGGCAGGACGAG (sense) and 5′-CAGTGGCGGCAGCAGGGGTGG (antisense). The green fluorescent protein (GFP) was subcloned from the AcGFP1-N vector (Clontech) sequence (Fig. 1A). The GFP sequence alone in the pRSET-A plasmid served as control. The nucleotide sequences of all constructs were verified by sequencing before protein production. The fusion protein from *E. coli* was purified to homogeneity by immobilized Ni-Affinity Chromatography (IMAC) (Bio-Rad), dialyzed and concentrated as suggested by the manufacturer. The identity of the purified Tat-Hsp27 fusion protein was confirmed by western blotting with anti-human Hsp27 antibody (Santa Cruz) (Fig. 1B). Protein transduction efficiency was measured by treating mouse H9c2 myocytes grown to 80% confluency in 6-well plates with varied concentrations of Tat-GFP fusion proteins for 4 h followed by flow cytometry for GFP levels. To assess possible toxic effects, cells seeded in 96-well plate were treated with different concentrations of Tat-Hsp27/GFP for 1 h and proliferation assessed using the CCK assay kit (Dojindo) according to the manufacturers' instructions.

Rescue after hypoxia stimulation

H9c2 cells at 80% confluency were treated with 1.0 μM Tat-Hsp27 fusion protein for 1 h and subsequently incubated for 48 h at 37°C, 94% N_2, 1% O_2, 5% CO_2 in a hypoxic chamber. Cell death was assayed after staining with Annexin V-PE by flow cytometry.

Rescue after chemical induction of apoptosis

Apoptosis was induced in mouse H9c2 myocytes with 10 nM staurosporine (Sigma Aldrich) for 3 h or 400 μM cobalt chloride ($CoCl_2$) (Sigma Aldrich) for 24 h, under serum-free conditions and then treated with different concentrations of Tat-Hsp27 for 1 h. Cellular apoptosis was determined by measurements of caspase 3 and 7 activity using the luminometric Caspase-Glo 3/7 assay (Promega) according to the manufacturer's instructions. Statistical analyses of the data was performed by one-way ANOVA, Significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

Construction and purification of Tat fusion proteins

The Tat peptide, a 11 amino acid signal sequence of the HIV-

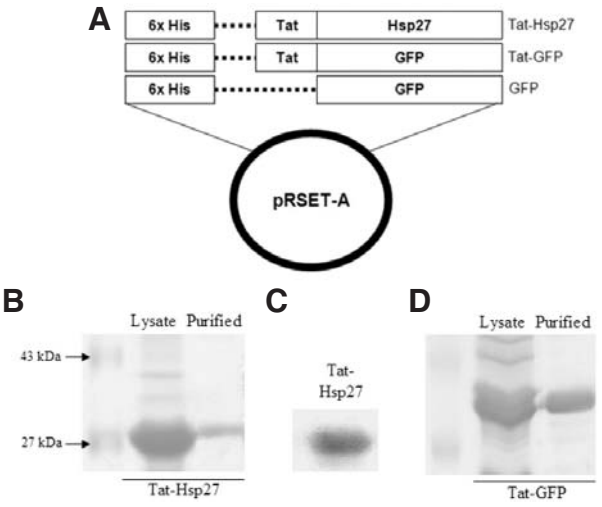

Fig. 1. Schematic representation of expression and purification of Hsp27- and GFP- fusion proteins. (A) the three expression vectors, pTat-Hsp27, pTat-GFP and pGFP were constructed as described in "Materials and Methods". (B) Analysis of purified Hsp27 fusion protein by SDS-PAGE Coomassie blue staining. The fusion proteins were purified by affinity chromatography on a nickel column. (C) Western blot analysis of purified Hsp27 fusion proteins using polyclonal antibody against human Hsp27. (D) Analysis of purified TAT-GFP fusion protein by SDS-PAGE Coomassie blue staining. The fusion protein was purified as done for Tat-Hsp27.

TAT protein is known to be responsible for direct delivery of the TAT protein into the cytoplasm of cells by mediating a process called 'transduction' across the cell membrane (Wadia and Dowdy, 2005). Tat transduced into cells by endocytic pathway mediated by clathrin or caveolae as an efficient cell translocation mediator (Fittipaldi et al., 2003; Richard et al., 2005). This property has been exploited to transduce several proteins into cells by engineering their expression as a Tat-fusion (Chauhan et al., 2007; Kumar et al., 2004; Kwon et al., 2007). We expressed the Hsp27 protein in fusion with the Tat peptide in the pRSET-A vector to enable entry upon addition to cells (Fig. 1A). The Tat-Hsp27 fusion protein was expressed to high levels with the expected molecular mass of approximately 30 kDa (Fig. 1B). As this vector allows expression of proteins in fusion with 6 His residues, we purified the protein to homogeneity using Nickel affinity chromatography. The purified protein was reactive in western blots with a human Hsp27- specific antibody confirming its identity (Fig. 1C). GFP was similarly expressed and purified in the same vector with or without the Tat peptide to serve as controls and for measuring the transduction efficiencies in further experiments (Fig. 1D).

Transduction of Tat-fusion proteins into cells

We determined the ability of the Tat transduction domain to transduce the fusion proteins by treating H9c2 cells with TAT-GFP and looked for GFP fluorescence by flow cytometry. Treated cells showed a clear increase in GFP fluorescence compared to untreated cells as shown in Fig. 2A. Transduction efficiencies as high as ~90% were observed in the myocardiac cell line, H9c2 with TAT-GFP. To ensure that the fluorescence was due to intracellular delivery of the protein, cells were observed by fluorescence microscopy. Clearly GFP fluorescence was detected throughout the cytoplasm as well as the nucleus in some of the cells treated with TAT-GFP (Fig. 2B, left panel).

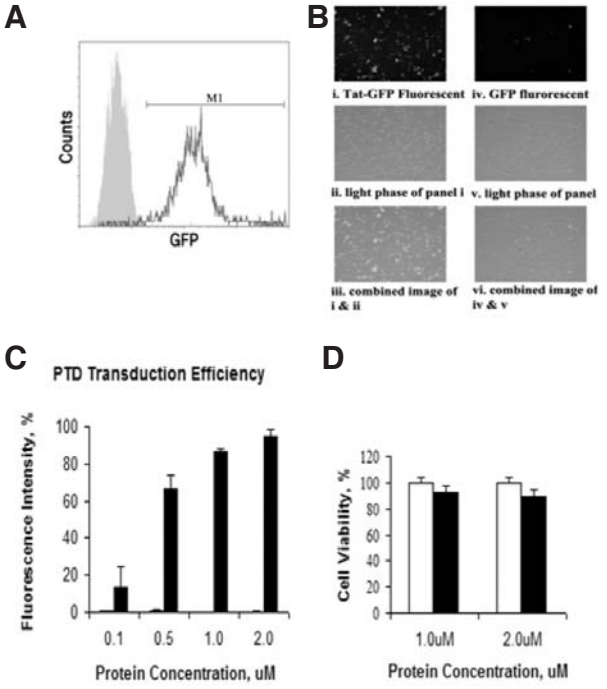

Fig. 2. Analysis of GFP fusion proteins transduction efficiency in H9c2 cells and the effect of Tat-Hsp27 transduction on cell viability. (A) Transduction efficiency comparison between GFP fusion proteins at different concentration into H9c2 cells with GFP only as control and the uptake was measured by flow cytometry. (B) H9c2 cells transduced with 1.0 μM of Tat-GFP, left panel and GFP, right panel. (C) Comparison of PTD-GFP fusion protein uptake by flow cytometry at 1.0 μM, % fluorescent intensity was calculated in comparison to untreated cells. Closed bar, Tat-GFP; open bar, GFP. (D) H9c2 cells were treated with different concentrations of Tat-Hsp27. Cell viabilities were estimated using the CCK assay. Closed bar, no treatment; open bar, Tat-Hsp27 treatment.

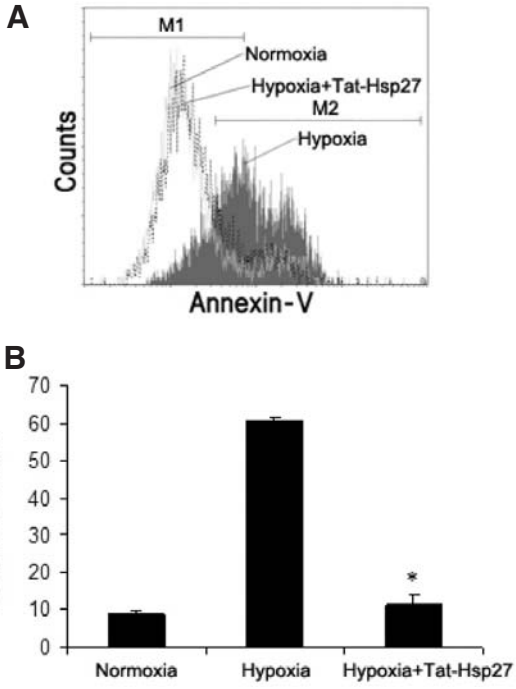

Fig. 3. Analysis effect of Tat-Hsp27 fusion proteins on hypoxia induced apoptosis. 1.0 μM Tat -Hsp27 treated H9c2 cells were subjected to hypoxia for 48 h. Cells were then stained with Annexin V and analyzed by flow cytometry. (A) Analysis of FACS from cells incubated in the condition of normoxia (opened line), hypoxia (closed line) and hypoxia + Tat-Hsp27 (dashed line). Gate 2 (M2) in the histogram depicts percentage of apoptotic cells while gate 1 (M1) are live cells. (B) Average values for duplicate experiments are normoxia = 9%, hypoxia = 61%, and hypoxia + Hsp27 = 12%.

This is supportive of the fact that the TAT peptide is also a nuclear localization signal and can mediate nuclear entry of transduced proteins (Chauhan et al., 2007). No fluorescence was visible in any of the cells treated with similar amounts of control GFP protein missing the Tat tag (Fig. 2B, right panel) confirmed that only the TAT fusion protein was capable of transducing across the cell. Our result confirms other studies that PTD is able to deliver proteins across the cell membrane efficiently into cells (Schwarze et al., 1999).

Flow cytometric analysis revealed that transduction of Tat-GFP was dose-dependent; with good levels of fluorescence intensity and good numbers of cells transduced at 1.0 μM protein concentration (Fig. 2C). Not much of an increase in transduction efficiency occurred from 1 to 2 μM concentrations (Fig. 2C). The efficiency of transduction of TAT-fusion proteins appears to vary depending on the cell type with as little as 140 nM of protein required to fully transduce Jurkat cells as opposed to 2 μM for HeLa cells, possibly due to varied levels of surface heparin sulphate (Mai et al., 2002). However as our experiments with Tat-GFP protein provided a good visual estimate of transduction efficiencies (≥ 80%), we decided to use the Tat-Hsp27 fusion protein in further experiments at a protein concentration of 1.0 μM.

To ensure that the Tat-Hsp-27 protein would not affect the viability of cells at this concentration, H9c2 cells were treated with Tat-Hsp27 and cell viability measured using the CCK cytotoxicity assay. Treated cells showed no signs of cytotoxicity with a concentration of 1 μM Tat-Hsp27 with > 95% of the cells remaining viable under these conditions (Fig. 2D). Thus Tat-Hsp27 treatment is non-toxic to cells under the experimental conditions. Although some reports use dextran sulfate for enhanced transduction efficiency of TAT in some cells (Mai et al., 2002), we never found that necessary as we always observed ~90% transduction efficiency with 1 μM Tat-Hsp27 in H9c2 cells.

Cells rescue from hypoxic apoptosis

The protective effect of Tat-Hsp27 fusion proteins against cellular apoptosis due to hypoxia was assessed by pre-treating myocytes, H9c2 cells with 1.0 μM Tat -Hsp27 an hour before subjecting them to hypoxia for 48 h. Non-treated cells cultured under normoxic conditions served as control. Exposure of H9c2 cells to hypoxia resulted in > 80% increase in apoptosis in comparison to cells under normoxic conditions (Fig. 3A). Pre-treatment with Tat-Hsp27 fusion proteins however resulted in a significant decrease of dying cells to about 50% (which amounts to approximately a 80% inhibition of apoptosis after normalizing for transduction efficiencies) indicating that introduction of Hsp27 into cells rescued a considerable proportion of cells from a death fate (Fig. 3B). These results are consistent with other studies of Hsp on cell apoptosis. Other heat shock proteins such as Hsp70 and Hsp 90 also showed a dramatic reduction of ischemic cell death both *in vitro* and *in vivo* (Martin et al., 1997; Radford et al., 1996; Trost et al., 1998). Hsp27 itself has

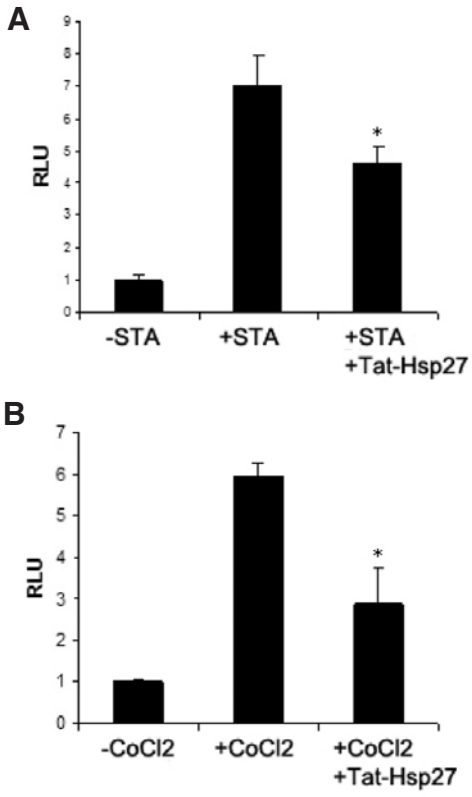

Fig. 4. Effect of Tat-Hsp27 in reversing apoptosis. H9c2 cells were pretreated with 1.0 μM Tat-Hsp27 1 h prior to induction (A) mitochondria-dependent (intrinsic) apoptosis by staurosporine (STA) treatment for 3 h or (B) mitochondria-independent (extrinsic) apoptosis by cobalt chloride (CoCl2) treatment for 24 h. Cells were analyzed with the Caspase-Glo 3/7 assay and relative luminescence (RLU value) is indicated. The asterisk indicates significant difference. -STA, without staurosporine treatment; +STA, with staurosporine treatment; STA+ Tat-Hsp27, Tat-Hsp27 treatment in staurosporine induced apoptosis group; -CoCl2, without cobalt chloride treatment; +CoCl2, with cobalt chloride treatment; CoCl2+Tat-Hsp27, Tat-Hsp27 treatment in cobalt chloride induced apoptosis group, *P < 0.05 in the Tat-Hsp27 treated groups under both conditions.

been used to protect cell death from vascular disease (Ferns et al., 2006).

Many other antiapoptotic proteins including FNK (a Bcl-x(L) derivative), apoptosis repressor with caspase recruitment domain (ARC) or beta-galactosidase (beta-gal) have similarly been conjugated to Tat and used for protection from apoptosis *in vitro* as well as *in vivo* (Arakawa et al., 2007; Gustafsson et al., 2002). In fact the Hsp-27 itself was found to have a beneficial role in a rat model of reperfusion heart injury (Kwon et al., 2007). All these experiments showed significant therapeutic efficacy of anti-apoptosis proteins in suppression of myocardial apoptosis following ischemia/reperfusion. However the method of action is not elucidated in any of these studies.

Elucidation of Hsp27 function in intrinsic/extrinsic apoptotic pathways

To determine whether the Hsp27 functions in the mitochondria-dependent, intrinsic apoptotic pathway, we examined the caspase-3 activity induced in H9c2 cells treated with staurosporine, which is known to trigger the intrinsic pathway (Columbaro et al., 2001; Yue et al., 1998). Pre-treatment of H9c2 cells with 1.0 μM of Tat-Hsp27 an hour before the addition of 10 nM staurosporine resulted in a modest reduction of caspase-3 activity (approximately 35%, $P < 0.05$) in comparison to control staurosporine-treated H9c2 cells (Fig. 4A). Another group reported that the survival protein Bcl-2 inhibits cell apoptosis induced by staurosporine, serum, and glucose deprivation and simulated ischemia (Mayorga et al., 2004). Our result thus indicated that Hsp27 may play a moderate role unlike Bcl-2, in reversing the intrinsic pathway. This anti-apoptotic activity has been elucidated by other groups that Hsp27 indeed directly bind to cytochrome C and co-precipitated with cytochrome C in mouse, rat and human cells (Garrido et al., 1999; Pandey et al., 2000; Paul et al., 2002; Whitlock et al., 2005). Therefore, we expect that Hsp27 prevent apoptosis by binding to cytochrome C and sequester caspase cascade. On the other hand, when the effect of Tat-Hsp27 was examined on cells treated with cobalt chloride, an agent which mimics hypoxic conditions by augmenting ROS formation through a mitochondria-independent pathway (Chandel et al., 1998; Zou et al., 2001), caspase-3 activity was reduced by more than > 50% ($p < 0.05$) when H9c2 were pretreated with Tat-Hsp27 an hour prior to the addition of 400 μM cobalt chloride (Fig. 4B).

Our data thus indicates that Hsp 27 can protect cells from apoptosis occurring by both the intrinsic and extrinsic apoptosis with a more dominant protective effect observed in inhibiting the Fas-mediated extrinsic apoptosis pathway. A recent report proposed that the mitochondrion is involved in both intrinsic and extrinsic pathways by serving as a control point for cross-talk between the two pathways since stimulation of the extrinsic pathway by death receptors leading to pro-caspase-8 activation (Yu et al., 2009). This results in the cleavage of an endogenous cellular protein, Bid, which in turn generates a pro-apoptotic fragment that translocates to the mitochondrion where it induces cytochrome C release (Li et al., 1998) further amplifying the caspase cascade. Hence it is quite plausible that although the effect of Hsp-27 appears to dominate in the extrinsic pathway, it may be a result of the cumulative effects in both pathways which warrants further investigation.

It is also noteworthy that several *in vivo* studies using TAT-fusion proteins do not document humoral or T cell responses to the TAT signal peptide (Kim et al., 1997; 2008; Lecoq et al., 2008) while, on the other hand, the full length TAT protein elicits dominant responses directed to aa 17-32, 33-48, and 65-80 (Kim et al., 2004). However, one cannot discount the potential immunogenicity of TAT-PTD especially upon repeated administration, which needs to be tested. If required, several modifications of the TAT peptide have been constructed and can be tested for reduced immunogenecity (Shaw et al., 2008). In our previous studies, we have observed that repeated treatment of a similar positively charged PTD (poly-9R) did not elicit innate or adaptive immune responses in immunocompetent mice. In the light of these observations, we feel that the TAT peptide can be used in *in vivo* applications with relatively low risks, especially when the situation does not demand prolonged repeated administration, as would be the case with TAT-Hsp fusion proteins (Kumar et al., 2007).

We demonstrated that using simple agents like staurosporine and cobalt chloride, can shed light on the degree of involvement of Hsp-27 in both pathways for its anti-apoptotic effect in rescuing cells from hypoxia-induced cell death. A deeper understanding of the mechanism of action of Hsp-27 would significantly contribute towards the applications where Tat-Hsp27 can find potential use to treat human disease caused by excessive apoptosis, for instance, treatment of disorders like the Lyell

syndrome, graft-versus-host disease (GVHD), multiple sclerosis, viral hepatitis and HIVs, thyroid diseases, alcoholic hepatitis, ulcerative colitis where the symptomatic effects are a direct consequence of cellular apoptosis.

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