

Sensitization of Mesothelioma Cells to Tumor Necrosis Factor–Related Apoptosis–Inducing Ligand–Induced Apoptosis by Heat Stress via the Inhibition of the 3-Phosphoinositide-Dependent Kinase 1/Akt Pathway

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Abstract

Heat stress may enhance the effect of apoptosis-inducing agents in resistant tumor cells. One such agent is the tumor necrosis factor–related apoptosis–inducing ligand (TRAIL), which has attracted intense interest for its ability to induce apoptosis in tumors without affecting nonmalignant cells. We therefore tested whether heat stress potentiates TRAIL-induced apoptosis in mesothelioma cells, its cell type being resistant to TRAIL alone. We found that heat stress enhanced the apoptosis caused by TRAIL but not by chemotherapy. To explain this potentiation, we found that heat stress decreased Akt phosphorylation via the dissociation of heat shock protein 90 (Hsp90) from its client protein 3-phosphoinositide-dependent kinase 1 (PDK-1), a major Akt kinase. The role of Hsp90 and the Akt pathway was confirmed by showing that inhibitors of Hsp90 and the phosphatidylinositol-3 kinase/Akt pathway reproduced the effect of heat stress on TRAIL-induced apoptosis and that the effect of inhibiting Hsp90 on TRAIL-induced apoptosis could be overcome by activating the Akt pathway with a constitutively active construct of the Akt kinase PDK-1. The effect of heat stress involved multiple steps of the apoptotic machinery. Heat stress potentiated the death receptor pathway, as shown by an increase in TRAIL-induced caspase 8 cleavage. Nonetheless, knockdown of Bid, the main intermediary molecule from the death receptor pathway to the mitochondria, inhibited the effect of heat stress, showing that mitochondrial amplification was required for potentiation by heat stress. In summary, these results support the novel concept that heat stress inhibits the Akt pathway by dissociating PDK-1 from its chaperone Hsp90, leading to potentiation of TRAIL-induced apoptosis in resistant malignant cells. [Cancer Res 2007;67(6):2865–71]

Introduction

The heat shock or stress protein response (SPR) has been classically defined as a highly conserved cellular defense mechanism characterized by the increased expression of stress proteins (1). This defense mechanism allows the cell to withstand a

subsequent insult that would otherwise be lethal, a phenomenon referred as “thermotolerance” or “preconditioning.” Nonetheless, there seems to be a two-phase response to stress. The later phase, taking several hours and requiring *de novo* protein synthesis, is involved in development of tolerance. There is also an early phase that takes place in the first minutes to hours and involves the dissociation of heat shock proteins as chaperones from key signaling molecules and the consequent inhibition of some signaling pathways (1). It is known that heat shock proteins can act as essential factors in numerous cell signaling pathways (2–4), and heat stress, by dissociating those chaperones from key signaling molecules, may affect key cellular responses in this early phase of SPR. Whereas heat stress may protect cells in the later phase, heat stress may enhance cell death in the early phase.

Heat is now used clinically in combination with i.p. or intrapleural chemotherapy to enhance the therapeutic response of various tumors (5), including mesothelioma (6), but the mechanism of this sensitization is unclear. For example, heat may act directly by increasing cellular toxicity or may act indirectly by increasing the penetration of the agents into the tumor. In studies using tumor necrosis factor–related apoptosis–inducing ligand (TRAIL) or fas ligand, there is evidence that heat can increase apoptosis via the death receptor pathway (7, 8). In these studies, heat stress promoted the ability of the death receptor ligand to induce cleavage of caspase 8, the initiator caspase. In some cells, such as the lymphocytes and Jurkat cells used in these studies (type I cells), such enhancement of death receptor signals can be sufficient to increase apoptosis. Other cells, including most solid tumor cells (type II cells), require amplification of death receptor signals at the mitochondria (9). Recruitment of the mitochondrial apparatus involves the caspase 8–dependent cleavage of an intermediary molecule, the BH3-only protein Bid, that then interacts with mitochondrial molecules to promote the release of apoptotic effectors. In this study, we have asked whether heat stress enhances apoptotic responses of malignant mesothelioma cells to TRAIL or to chemotherapy. We chose to study mesothelioma cells, which as type II cells require the mitochondrial apparatus for TRAIL-induced apoptosis, because they are derived from a tumor resistant to existing therapies (10, 11) and a tumor that may respond to heat stress clinically (6). In addition, like many tumors when studied *in vitro*, mesothelioma is resistant both to TRAIL and to chemotherapeutic agents, as shown by studies using cell lines (12) and actual tumor fragment spheroids (13). We focused on the human mesothelioma cell line M28, because in this line we have extensively studied the mechanism of sensitization to TRAIL-induced apoptosis by DNA damaging agents (14).

Note: M.H. Pespeni and M. Hodnett contributed equally to the work summarized in this article. V.C. Broaddus and J.F. Pittet contributed equally to this article.

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We report here that mesothelioma cells can be sensitized to TRAIL-induced apoptosis by heat stress, a sensitization that lasted for at least 12 h after the stress. Interestingly, heat stress did not enhance the apoptosis caused by several chemotherapeutic agents nor did heat stress sensitize nonmalignant mesothelial cells to TRAIL. We further report that this sensitization is due to the inhibition of the phosphatidylinositol-3 kinase (PI3K)/Akt pathway via a dissociation of heat shock protein 90 (Hsp90) from its client protein 3-phosphoinositide-dependent kinase 1 (PDK-1), a major kinase of Akt. Heat stress enhanced the TRAIL-induced cleavage of caspase 8 but also required an amplification step involving the mitochondria as shown by the need for Bid. These results support the novel concept that the dissociation of Hsp90 from its client protein PDK-1 observed early after heat stress is an important mechanism to explain how the activation of SPR facilitates apoptosis in cancer cells that are otherwise resistant to classic therapies.

Materials and Methods

Reagents. Human recombinant TRAIL (375-TL, histidine-tagged extracellular domain) was purchased from R&D Systems, Inc. (Minneapolis, MN) and used at a final concentration of 1.0 or 2.5 ng/mL. Anisomycin (Sigma, St. Louis, MO), an agent that sensitizes to TRAIL by toxic effects on ribosomes, was used at the final concentration of 25 ng/mL. Etoposide was purchased from Bedford Laboratories (Bedford, OH), dissolved in Me₂SO, and used at final concentration of 10 or 150 μg/mL. Gemcitabine (Eli Lilly, Indianapolis, IN) was used at the final concentration of 0.15 or 3 mmol/L. Cisplatin (SICOR Pharmaceuticals, Irvine, CA) was used at the final concentration of 40 or 400 μmol/L. The concentrations for the chemotherapeutic agents (etoposide, gemcitabine, and cisplatin) were chosen to represent, at the lower range, concentrations used in cell culture (14) and, at the higher range, those used in i.p. chemotherapy (5). LY294002, an inhibitor of the PI3K/Akt, was used at the concentration of 50 μmol/L and was obtained from Sigma.

Bid, Akt, phosphorylated Akt (p-Akt, both serine and threonine), phosphorylated p70S6K, caspase 8, and β-actin antibodies were obtained from Cell Signaling (Beverly, MA). C-FLIP antibody was obtained from Alexis Biochemicals (San Diego, CA). Hsp90 antibody was obtained from Stressgen (Victoria, Canada). Protein A-Sepharose and G-Sepharose beads were purchased from Amersham (Piscataway, NJ). Protein concentration of cell lysates was determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard. All other chemicals were purchased from Sigma.

Cell culture, SPR activation, and chemical treatments. Human malignant mesothelioma cell line M28 (from Dr. Brenda Gerwin, National Cancer Institute, NIH, Bethesda, MD) and primary human mesothelial cells were grown in standard media, as we have previously reported (14). Primary human mesothelial cells were cultured from ascites fluid from patients without infection or malignancy according to a protocol approved by the University of California San Francisco Committee on Human Research. The heat stress protocol was as follows. Subconfluent M28 cells were subjected to heat stress at 43°C for 60 min or kept at 37°C for 60 min in humidified incubators at 5% CO₂. After this 60-min period, all cells were placed back at 37°C for a recovery period of between 1 and 24 h before further treatments. The standard recovery was 1 h. Cells were exposed to TRAIL at a concentration of 1 or 2.5 ng/mL for the times indicated in the figure legends. For inhibition of Hsp90 function, M28 cells were exposed to 17-allylamino-17-demethoxygeldanamycin (17-AAG; 1–2 μg/mL) for 1 to 8 h.

Western blot analysis. Western blot analysis was done as described previously (14). After equal amounts of protein were loaded in each lane and separated by 10% SDS-PAGE, proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were blocked with 5% dry milk in PBS containing 0.1% Tween 20 and incubated with the primary antibody (1:1,000) overnight at 4°C and with horseradish peroxidase-

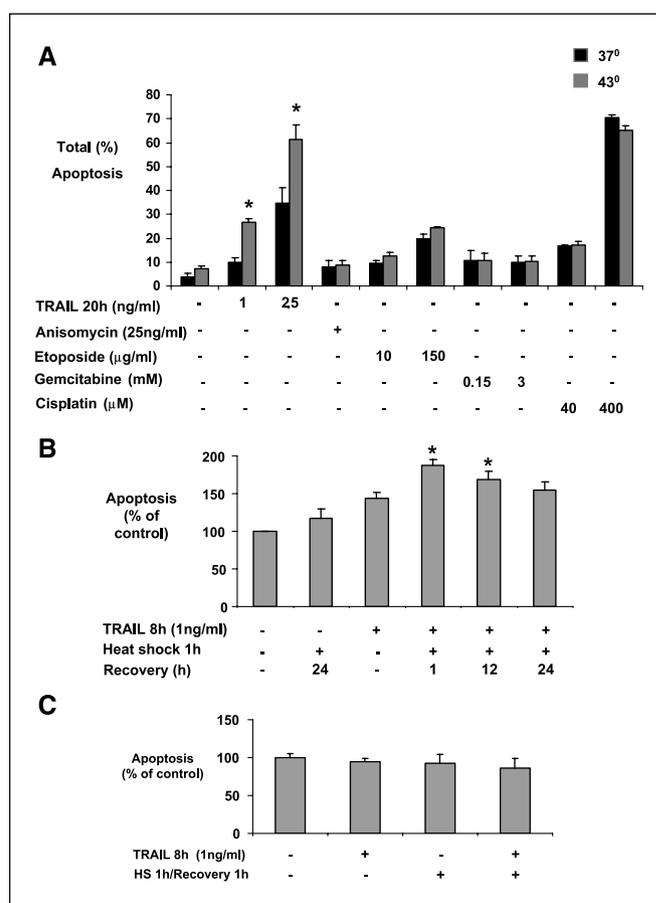


Figure 1. Heat stress potentiates apoptosis due to TRAIL, but not to chemotherapeutic agents, in malignant mesothelioma cells. **A**, heat stress (43°C for 60 min, recovery 60 min) enhances apoptosis due to TRAIL (1 or 2.5 ng/mL for 20 h) in M28 mesothelioma cells. Heat stress had no effect on apoptosis due to the ribotoxic agent anisomycin or to the DNA damaging agents etoposide, gemcitabine, or cisplatin. **B**, after activation of SPR by heat, the potentiation of apoptosis was maximal when TRAIL (1 ng/mL for 8 h) was added after a 1-h recovery and was still present after a 12-h recovery. **C**, in primary nonmalignant human mesothelial cells, heat stress (HS) had no effect on subsequent apoptosis due to TRAIL (1 ng/mL for 8 h). Columns, mean of at least three experiments done in triplicate; bars, SE. *, $P < 0.05$ versus TRAIL alone.

conjugated secondary antibody (1:2,000) for 1 h at room temperature. Proteins were visualized using chemiluminescence. Quantification was done using a digital image analysis system (NIH Image).

Coimmunoprecipitation for PDK-1 and Hsp90. Cells were washed thrice with PBS and a fourth time with PBS and aprotinase (10 mg/mL). Cells were then lysed with a lysis buffer [150 mmol/L NaCl, 2 mm CaCl₂, 10 mmol/L Tris-HCl (pH 7.5, TBS), 1% Triton X-100, 40 mmol/L NaMolyB, 10 units/mL aprotinase] supplemented with a cocktail of phosphatase and proteinase inhibitors (1 mmol/L vanadate, 10 mg/mL leupeptin, 10 mg/mL aprotinin, 1 mmol/L phenyl-methyl-sulfonyl fluoride). Immunoprecipitations were done by incubating the lysates with the primary antibody overnight at 4°C. Then, the cell lysates were incubated for 2 h at 4°C under continuous mixing with protein A-Sepharose and G-Sepharose beads, and the Sepharose-bound immune complexes were washed four times with the same lysis buffer and boiled in 2× Laemmli sample buffer. Detection of PDK-1 and Hsp90 was determined by Western blot, as described above.

RNA interference and transfection with constitutively active PDK-1. Knockdown of Bid or transfection with constitutively active PDK-1 was accomplished using nucleofection to introduce small interfering RNA oligonucleotides (14) or the constitutively active PDK-1 (gift from Dr. F. Liu, University of Texas Health Science Center) into cells. The constitutively active PDK-1 mutant was made by replacing the alanine in position 280

with a valine (15). This mutant has been shown to increase PDK-1 kinase activity and bypass the requirement for PI3K in transducing upstream signals to Akt (15). Briefly, 5×10^6 M28 cells were pelleted and resuspended in 100 μ L of solution V (Amaxa Biosystems, Cologne, Germany) containing 3 μ g of Bid small interfering RNA (antisense strand: UAU UCC GGA UGA UGU CUU CdTT; Xeragon Inc., Huntsville, AL) or 3 μ g of PDK-1^{A280V}. The Bid small interfering RNA had been previously shown to be specific for Bid (14). An irrelevant small interfering RNA (antisense strand: ACG UGA CAC GUU CGG AGA A dTT) was used as a control. This suspension was transferred to a sterile cuvette and nucleofected using program T-20 on a Nucleofector II device (Amaxa Biosystems). Cells were recovered for 30 min in an Eppendorf containing serum-free RPMI medium before being plated. Cells were studied 48 h postnucleofection.

Measurement of apoptosis. Apoptosis was quantitated using FITC-Annexin V (BD Biosciences, Palo Alto, CA), as described before (14). The extent of apoptosis and the lack of necrosis (<5%) were confirmed in selected experiments by direct morphologic analysis of cells stained with acridine orange and ethidium bromide, as reported before (14).

Statistics. All the data are summarized as mean \pm SE. One-way ANOVA, with Tukey's test for post hoc analysis, or the Fisher's exact *t* test was used to compare experimental with control groups, using GraphPad Prism version 4.0 (GraphPad Software, Inc., San Diego, CA). A *P* value of <0.05 was considered statistically significant.

Results

Heat stress increases TRAIL-induced apoptosis. M28 mesothelioma cells are refractory to induction of apoptosis by many

chemotherapeutic agents or by TRAIL given alone. These cells were, therefore, used to test whether heat stress would sensitize them to apoptotic agents. SPR activation by heat (43°C for 60 min, recovery 60 min) led to a significant increase in apoptosis due to TRAIL (1 or 2.5 ng/mL for 20 h; Fig. 1A). This potentiation was not observed when M28 cells were heat stressed and then exposed to several chemotherapeutic agents that are used clinically in combination with heat stress, such as etoposide, gemcitabine, or cisplatin (Fig. 1A).

To determine whether heat stress permanently sensitized M28 cells to TRAIL or whether this sensitization was time dependent, apoptosis was measured after increasing recovery times from heat stress. We found that, in the malignant cells, the potentiation by heat stress was maximal when TRAIL was given after a 1-h recovery from heat stress but was still present after a 12-h recovery (Fig. 1B). At 24 h of recovery, the level of apoptosis had returned to that seen with TRAIL alone. Thus, the potentiation of TRAIL-induced apoptosis by heat stress is a transient phenomenon. In contrast, for nonmalignant human mesothelial cells, heat stress, even after 1 h of recovery, failed to potentiate the effect of TRAIL (Fig. 1C).

Heat stress inhibits the Akt pathway. Because the Akt pathway, constitutively active in M28 cells (16), is a known survival pathway able to counteract TRAIL-induced apoptosis (17), we investigated the effect of heat stress on Akt phosphorylation and activation. Heat stress significantly attenuated Akt phosphorylation at serine 473

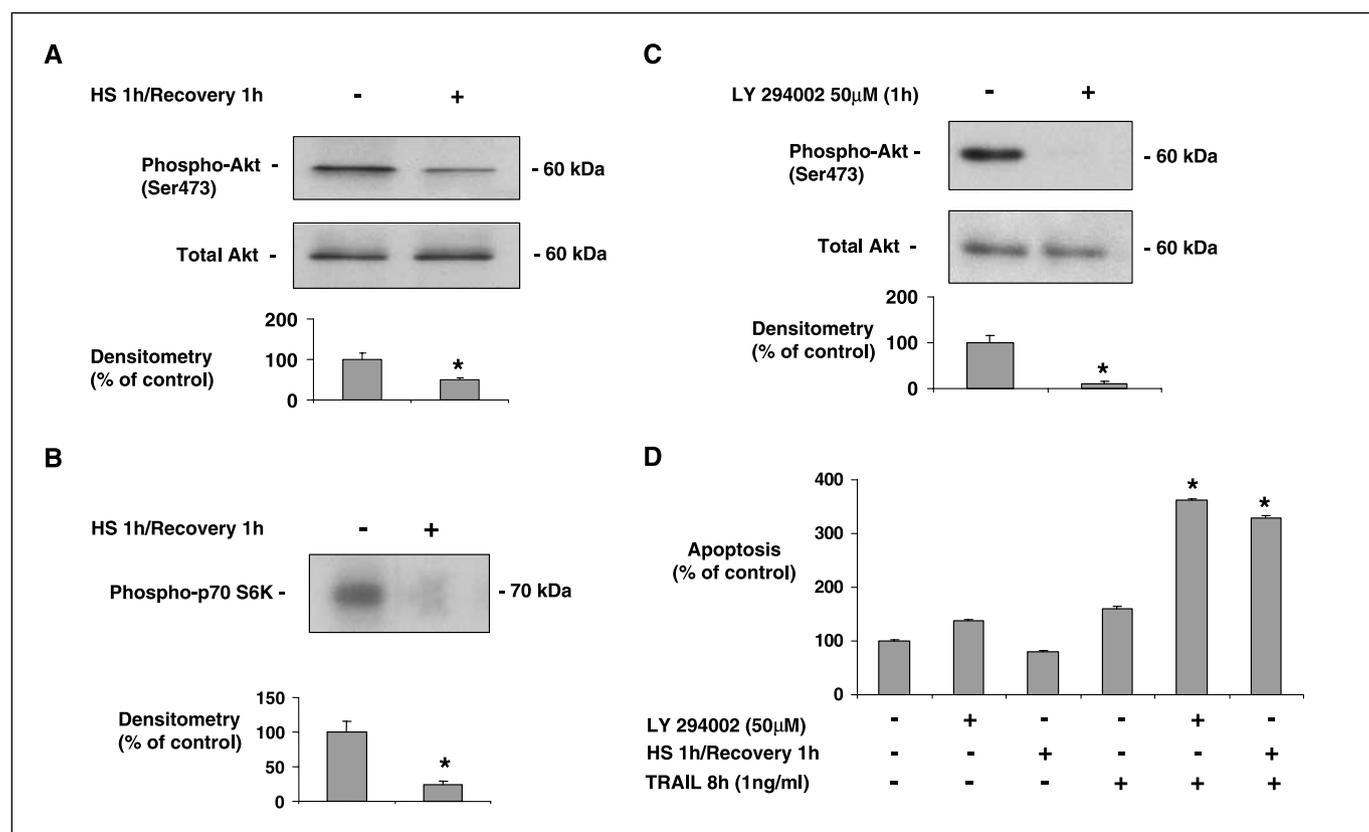


Figure 2. Role of PI3K/Akt in the potentiation of TRAIL-induced apoptosis by heat stress. *A*, the high baseline expression of p-Akt in M28 mesothelioma cells [phospho-Akt (Ser473)] is decreased at the end of heat stress. One representative experiment. Columns, mean of three experiments; bars, SE. *, *P* < 0.05 versus control. *B*, activation of SPR by heat also significantly inhibits the phosphorylation of p70S6K, a kinase downstream of Akt, measured at the end of heat stress. One representative experiment. Columns, mean of three experiments; bars, SE. *, *P* < 0.05 versus control. *C*, LY294002 (50 μ mol/L for 1 h) significantly inhibits the high constitutive Akt phosphorylation in M28 mesothelioma cells. One representative experiment. Columns, mean of three experiments; bars, SE. *, *P* < 0.05 versus control. *D*, TRAIL alone (1 ng/mL for 8 h) induces minimal apoptosis in M28 mesothelioma cells. However, pretreatment with LY294002 (50 μ mol/L for 1 h) or heat stress enhances the effect of TRAIL (1 ng/mL for 8 h). Columns, mean of three experiments done in triplicate; bars, SE. *, *P* < 0.05 versus TRAIL alone.

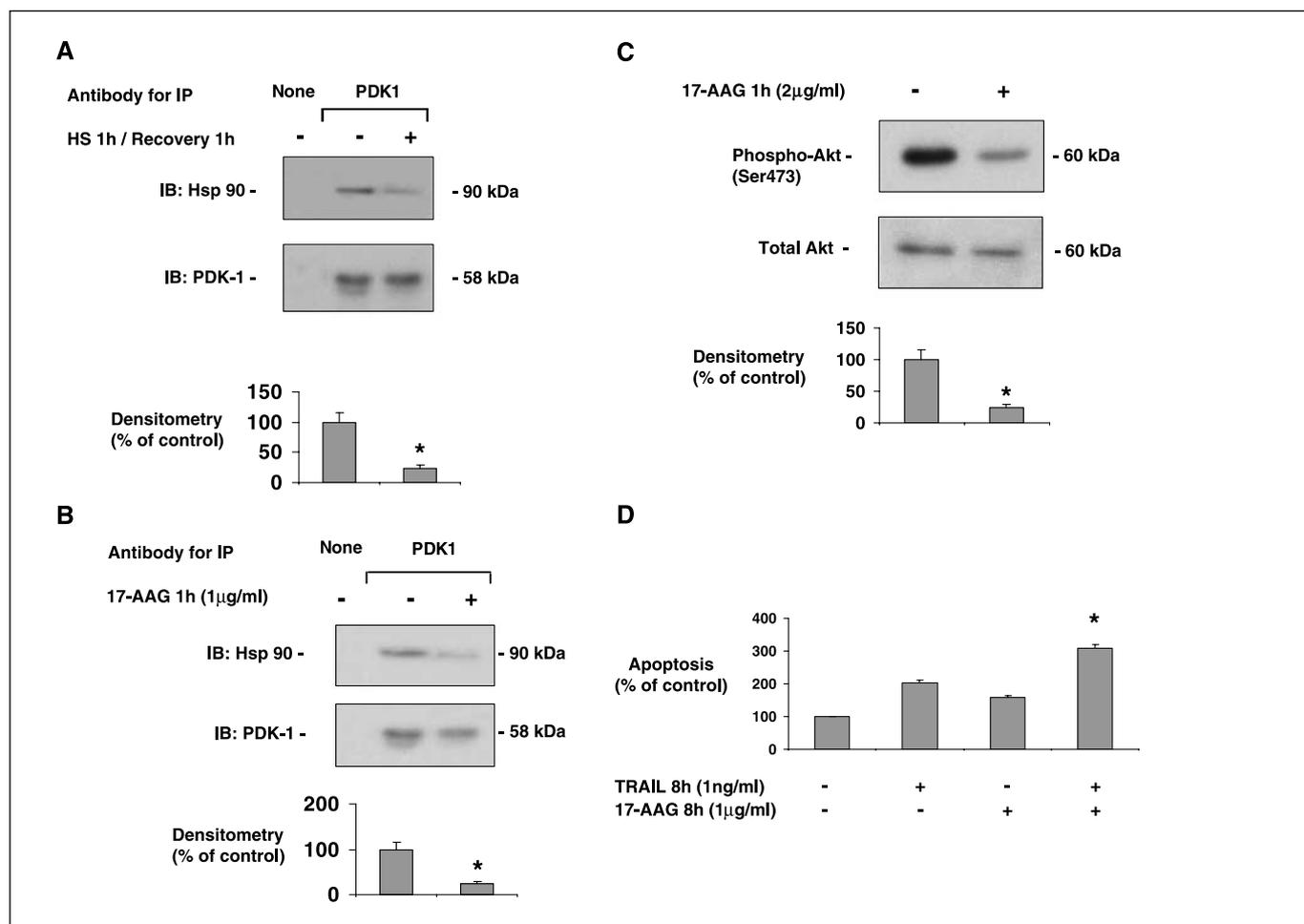


Figure 3. Heat stress and 17-AAG dissociate Hsp90 from PDK-1. **A**, M28 mesothelioma cells exposed to heat stress (43°C for 60 min, recovery 60 min) were subjected to lysis and immunoprecipitation (IP) with an antibody specific for PDK-1. The resultant immunoprecipitates were then examined via immunoblotting (IB) for Hsp90 and PDK-1. At baseline, PDK-1 associated with Hsp90. At 1 h after heat stress, the PDK-1 has mostly dissociated from Hsp90. In the absence of antibody, no nonspecific association is seen. One representative experiment of three. *Columns*, mean of at least three experiments; *bars*, SE. *, $P < 0.05$ versus no heat stress control. **B**, 17-AAG leads to a dissociation of Hsp90 from the PDK-1. M28 cells were exposed to 17-AAG (1 μ g/mL for 1 h) and subjected to lysis and immunoprecipitation with an antibody specific for PDK-1. The resultant immunoprecipitates then were examined via immunoblotting for Hsp90 and PDK-1. One representative experiment. *Columns*, mean of three experiments; *bars*, SE. *, $P < 0.05$ versus controls. **C**, pretreatment with 17-AAG (2 μ g/mL for 1 h) significantly decreases p-Akt. One representative experiment. *Columns*, mean of three experiments; *bars*, SE. *, $P < 0.05$ versus controls. **D**, TRAIL alone (1 ng/mL for 8 h) induces minimal apoptosis in M28 mesothelioma cells. However, 17-AAG (1 μ g/mL) enhances the apoptosis due to TRAIL (1 ng/mL for 8 h). *Columns*, mean of three experiments done in triplicate; *bars*, SE. *, $P < 0.05$ versus TRAIL alone.

(Fig. 2A) and at threonine 308 (data not shown). The decrease in p-Akt was still present 12 h after heat but not after 24 h (data not shown). As further evidence of the inhibition of Akt activity, SPR activation also decreased the phosphorylation of p70S6K, a downstream member of the Akt pathway (Fig. 2B). To confirm the role of the Akt pathway in preventing the apoptosis of mesothelioma cells, M28 cells were treated with LY294002 (50 μ mol/L), an inhibitor of the PI3K/Akt pathway. LY294002 inhibited the phosphorylation of Akt (Fig. 2C) and increased TRAIL-induced apoptosis to the same level as observed after heat stress (Fig. 2D).

Heat stress inhibits the Akt pathway via the dissociation of Hsp90 from its client protein PDK-1, a major kinase for Akt. We have previously reported that the dissociation of Hsp90 from its client proteins is one of the mechanisms by which SPR activation inhibits cell transduction pathways (4). We therefore tested the hypothesis that activation of SPR by heat would dissociate Hsp90 from Akt or from its upstream kinases. We were unable to identify an association between Hsp90 and Akt itself but were able to show

an association between Hsp90 and PDK-1, a major kinase for Akt and a known client protein of Hsp90 (18). Using immunoprecipitation, we found that Hsp90 formed a complex with PDK-1 in the unstressed cell that was not observed after the cells were heat stressed (Fig. 3A). To explore the role of the dissociation of Hsp90 from PDK-1 in inhibiting the Akt pathway and potentiating TRAIL-induced apoptosis, we tested the effects of an inhibitor of Hsp90 ATPase activity, 17-AAG. Similarly to heat stress, 17-AAG led to a dissociation of Hsp90 from PDK-1 (Fig. 3B), caused an inhibition of Akt phosphorylation at serine 473 (Fig. 3C) and at threonine 308 (data not shown), and potentiated the apoptosis induced by TRAIL (Fig. 3D). These data using 17-AAG support the hypothesis that the binding of Hsp90 to the Akt kinase PDK-1 is important in the activation of the Akt signaling pathway and that the activation of this pathway antagonizes TRAIL-induced apoptosis. If this is true, then using a constitutively active PDK-1 kinase that does not require Hsp90 binding for its activity should abrogate the effect of 17-AAG on Akt phosphorylation and apoptosis. M28 cells were thus

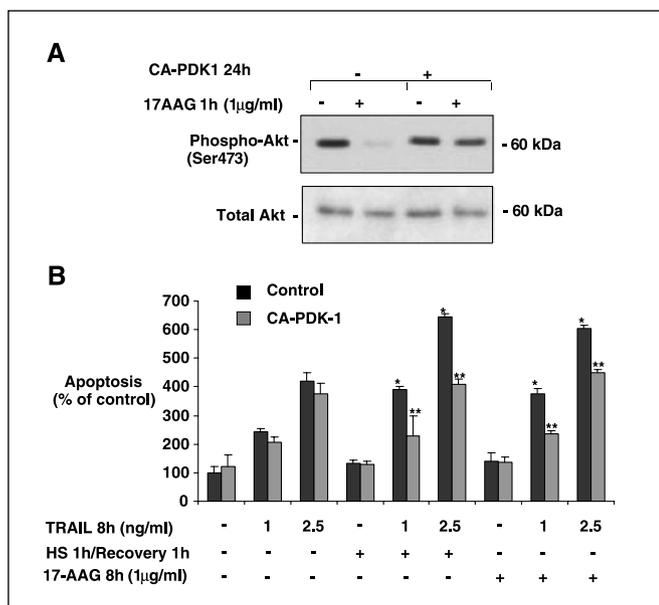


Figure 4. A constitutively active PDK-1 (*CA-PDK-1*) maintains Akt activity, thereby blocking the ability of heat stress or 17-AAG to potentiate TRAIL-induced apoptosis. *A*, M28 mesothelioma cells were subjected to mock transfection or transfection with a constitutively active PDK-1 plasmid. Forty-eight hours later, the cells were exposed to 17-AAG (1 μg/mL for 1 h). The active PDK-1 was able to prevent the decrease in p-Akt seen with 17-AAG. *B*, transfection of M28 cells with constitutively active PDK-1 prevented the ability of heat stress or 17-AAG (1 μg/mL for 8 h) to potentiate TRAIL-induced apoptosis. *Columns*, mean of three experiments done in triplicate; *bars*, SE. *, $P < 0.05$ versus TRAIL alone; **, $P < 0.05$ versus controls without constitutively active PDK-1.

transfected with a constitutively active PDK-1 that successfully maintained the level of p-Akt and prevented its decrease by 17-AAG (Fig. 4A). Significantly, the constitutively active PDK-1 inhibited the ability of heat stress or 17-AAG to potentiate TRAIL-induced apoptosis (Fig. 4B). Thus, taken together, these results indicate that the dissociation of Hsp90 from its client protein PDK-1 observed early after SPR activation is an important mechanism by which SPR inhibits the PDK-1/Akt pathway and, thus, contributes to the apoptotic synergy between heat stress and TRAIL in malignant cells.

Heat stress increases TRAIL-induced caspase 8 cleavage and uses Bid and mitochondrial amplification to potentiate TRAIL-induced apoptosis. Further studies were undertaken to identify steps in the TRAIL apoptotic pathway that were enhanced by the heat stress. After heat stress, TRAIL induced an increased cleavage of both caspase 8 and the caspase 8 target Bid (Fig. 5A and B). Due to the increase in TRAIL-induced caspase 8 and Bid cleavage caused by heat stress, we then examined whether this effect was associated with a decrease in c-FLIP expression, as has previously been reported (7, 8). We found that SPR activation by heat led to a decrease in the expression of c-FLIP (Fig. 5C). In type I cells, such as lymphocytes, caspase 8 cleavage due to death receptor signals can be sufficient to mediate apoptosis. Thus, we then asked whether the increase in death receptor signaling and caspase 8 cleavage was sufficient to explain the increase in TRAIL-induced apoptosis seen after heat stress. We reasoned that if the increase in caspase 8 cleavage fully accounted for the effect of SPR activation on TRAIL-induced apoptosis by activating downstream caspases and initiating apoptosis as in type I cells, then mitochondrial amplification would not be necessary. If so, then

knockdown of Bid, the key intermediary protein cleaved by caspase 8 cleavage and mediating mitochondrial amplification of TRAIL signals, would have little effect on the ability of heat stress to enhance apoptosis. Instead, we found that Bid depletion by RNA interference significantly reduced the effect of heat stress on

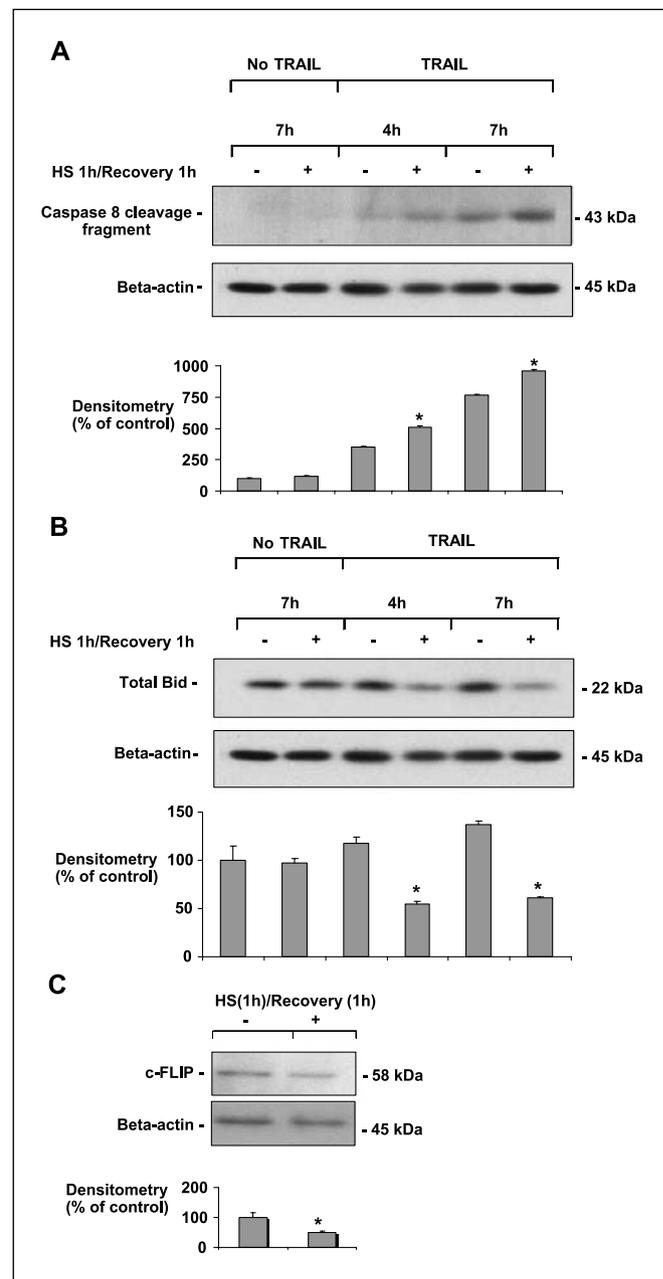


Figure 5. Heat stress increases TRAIL-induced caspase 8 and Bid cleavage and decreases the expression of c-FLIP. *A* and *B*, M28 mesothelioma cells were exposed to heat stress (43°C for 60 min, recovery 60 min) followed by no treatment for 7 h or TRAIL (1 ng/mL) for 4 or 7 h. Heat stress led to an increase in TRAIL-induced caspase 8 cleavage as judged by an increased detection of the large 43-kDa fragment of caspase 8. In addition, heat stress induced an increase of the TRAIL-induced cleavage of Bid, as judged by a decrease in whole Bid. Actin was used as a standard to confirm equivalent protein loading. One representative experiment. *Columns*, mean of at least three experiments; *bars*, SE. *, $P < 0.05$ versus TRAIL alone. *C*, heat stress (43°C for 60 min, recovery 60 min) decreases expression of c-FLIP. One representative experiment. *Columns*, mean of at least three experiments; *bars*, SE. *, $P < 0.05$ versus controls.

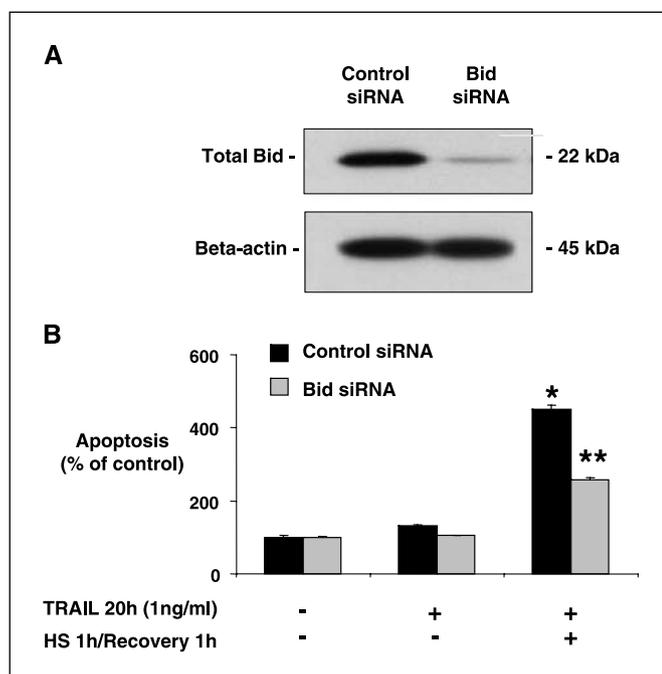


Figure 6. Heat stress requires Bid for full potentiation of TRAIL-induced apoptosis. *A*, M28 mesothelioma cells were transfected with either random or Bid-specific small interfering RNAs, showing almost complete inhibition of Bid protein expression by Bid-specific small interfering RNA at 48 h after transfection. *B*, M28 cells transfected 48 h previously with Bid-specific or random small interfering RNA were exposed to heat stress and TRAIL. The absence of Bid inhibited the effect of heat stress on subsequent TRAIL-induced apoptosis. Columns, mean of at least three experiments done in triplicate; bars, SE. *, $P < 0.05$ versus cells transfected with the random small interfering RNA.

TRAIL-induced apoptosis (Fig. 6*A* and *B*). The contribution of Bid to the effect of SPR indicates the need for mitochondrial amplification in the ability of SPR to potentiate TRAIL-induced apoptosis in these type II cells.

Discussion

Activation of SPR with heat is currently used as adjuvant therapy in combination with irradiation or chemotherapeutic agents for the treatment of several types of cancers in humans (5) including malignant mesothelioma (6), a tumor resistant to current therapies (10, 11). However, the underlying mechanisms for additive or synergistic effects of heat stress in cancer therapy are unknown. At the same time, TRAIL has become an attractive strategy for inducing apoptosis in cancer cells (19), although many cancer cells, such as mesothelioma, are resistant to TRAIL when used alone (20). Thus, the first objective of these studies was to determine whether activation of SPR by heat would alter apoptosis due to either TRAIL or chemotherapeutic agents in a mesothelioma cell line, one that is well studied for mechanisms of apoptotic synergy (14). In the present study, we found that these mesothelioma cells can be sensitized to TRAIL-induced apoptosis by heat stress, whereas there was no effect of heat on the apoptosis caused by chemotherapeutic agents. This sensitization to apoptosis was specific for the malignant mesothelioma cells with no sensitization seen for nonmalignant mesothelial cells. Interestingly, the effect of heat stress was predominantly proapoptotic and was not overwhelmed by the SPR-induced expression of heat shock proteins that exert antiapoptotic effects, such as Hsp72 (expressed 8 h after heat stress

treatment in M28 cells, data not shown; ref. 21). These findings suggest that the proapoptotic effects of heat stress dominate, at least in the first 12 h, over its potential antiapoptotic effects. The lack of effect of heat on the apoptosis due to chemotherapeutic agents suggests that beneficial effects of heat stress on responses to chemotherapy may be derived from mechanisms other than apoptosis. It further suggests that heated approaches could be effective in combination with TRAIL or TRAIL receptor agonists, now in phases I and II of clinical trials (22).

It has previously been reported that PI3K/Akt is a critical survival pathway for cancer cells (17) and is constitutively activated in many cancers including mesothelioma (16, 23). Our results have shown for the first time that heat stress can significantly attenuate Akt activity and, thereby, enhance apoptotic responses to TRAIL. The inhibitory effect of heat stress on Akt phosphorylation was present for at least 12 h after the end of heat stress, but was lost by 24 h, at the same time course over which heat stress potentiated to TRAIL-induced apoptosis. The importance of the inhibition of the Akt pathway by heat stress on TRAIL-induced apoptosis was underscored by (a) the ability of PI3K/Akt inhibitors to enhance TRAIL apoptosis and (b) the ability of restoration of Akt phosphorylation with a constitutively active PDK-1 construct to ablate the effect of heat stress in these mesothelioma cells. In summary, heat stress was shown to inhibit an important survival pathway in these cells, at least for 12 h in the early phase of SPR. Due to the widespread importance of the Akt pathway in cancer survival, these findings may apply not only to mesothelioma but also to other tumors that rely on Akt survival signals for resistance to apoptotic stresses, such as TRAIL.

We believe we have also identified the mechanism by which heat stress inhibits the PI3K/Akt pathway. In previous studies, we reported that activation of the heat shock response inhibited the NF- κ B pathway by dissociating Hsp90 from its client protein I κ B kinase, a major mediator of this pathway (4). In these studies of the PI3K/Akt pathway, we show that heat stress similarly causes the dissociation of Hsp90 from its known client protein PDK-1, a major kinase for Akt (18, 24). We confirmed the importance of Hsp90 in the effect of heat stress by demonstrating that the potentiation of TRAIL-induced apoptosis on mesothelioma cells could be reproduced by using an inhibitor of Hsp90, 17-AAG. Indeed, the inhibition of Hsp90 by 17-AAG has been shown to sensitize other cancer cells to TRAIL-induced apoptosis (25, 26), possibly via a mechanism similar to the one we outline here, by inhibition of key survival pathways. Furthermore, the effect of Hsp90 inhibition on TRAIL-induced apoptosis could be overcome by activating the Akt pathway with a constitutively active construct of the Akt kinase PDK-1. Normally, then, Hsp90 functions as a positive regulator of multiple cell transduction pathways, allowing the client protein to be folded in the correct conformation and preventing its degradation by the proteasome (27). We have learned that heat stress can disrupt this normal supportive function of Hsp90, thereby down-regulating various survival pathways, such as NF- κ B and PI3K/Akt. In summary, we show that heat stress causes the potentiation of TRAIL-induced apoptosis in mesothelioma cells by interfering with the normal binding of Hsp90 with PDK-1, leading to the inhibition of the Akt pathway.

Heat stress enhanced apoptosis at more than one step. After heat stress, the death receptor pathway was enhanced as shown by early evidence of increased TRAIL-induced caspase 8 cleavage that was associated with the inhibition of c-FLIP protein expression, an Akt target that inhibits caspase 8 cleavage (7, 8).

We asked whether the increased signaling via the death receptor pathway could be the sole explanation for the effect of heat stress, as was likely the case in studies of type I cells (7, 8). Heat stress could enhance the death receptor pathway sufficiently so that a mitochondrial step would no longer be necessary and our type II cells would operate more as type I cells. However, we found that Bid and its interaction with the mitochondria were necessary for full potentiation by heat stress. Mitochondria may have been recruited in the response to heat stress, either due to the increased cleavage of Bid after heat stress or due to additional effects of heat stress on the mitochondrial apoptotic machinery lowering its threshold to respond to truncated Bid. We suspect the latter because, in our previous studies, these cells failed to respond to TRAIL-induced cleaved Bid until the mitochondrial threshold was lowered by DNA damaging agents (14). Indeed, heat stress could be expected to lower the apoptotic threshold of the mitochondrial machinery in several ways. First, heat stress inhibits Akt, and Akt is known to support cell survival by actions at the level of the mitochondria, including the phosphorylation and inactivation of the proapoptotic molecule Bad and the promotion of glucose utilization (17). In addition, heat stress may directly activate proapoptotic proteins, such as Bax and Bak, involved in the mitochondrial pathway of apoptosis (28). Taken together, it is likely that SPR activation sensitizes cancer cells to

TRAIL-induced apoptosis not only via the enhancement of the extrinsic death receptor pathway but also via enhancement of the intrinsic mitochondrial pathway.

In this article, we have explored how SPR activation sensitizes mesothelioma cells to TRAIL-induced apoptosis. We have shown that this sensitization is due to the inhibition of the PDK-1/Akt complex via the dissociation of Hsp90 from its client protein PDK-1, leading to enhancement of the extrinsic death receptor pathway, as shown by an increase in TRAIL-induced caspase 8 cleavage. In these type II cells, heat stress was also shown to involve the intrinsic mitochondrial pathway. Because heat is used clinically in cancer therapy, these studies may lead to the application of SPR strategies (by heat stress or by chemical inhibitors of Hsp90) to future studies of TRAIL therapy. In summary, this study indicates how a combination of different proapoptotic and sublethal signals can offer an effective strategy for treating cancers refractory to conventional therapies.

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Sensitization of Mesothelioma Cells to Tumor Necrosis Factor–Related Apoptosis–Inducing Ligand–Induced Apoptosis by Heat Stress via the Inhibition of the 3-Phosphoinositide-Dependent Kinase 1/Akt Pathway

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