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Hsp90 inhibitors, GA and 17AAG, lead to ER stress-induced apoptosis in rat histiocytoma

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ABSTRACT

Heat shock protein 90 (Hsp90) is a major molecular chaperone that plays an essential role in the maintenance of several signaling molecules, most of which are oncogenic kinases. Hsp90 inhibition by specific inhibitors leads to destabilization and loss of activity of such proteins, thereby leading to inhibition of multiple signaling cascades. Due to this, Hsp90 has emerged as an important target for the treatment of cancer. Inhibition of Hsp90 has been reported to induce apoptosis in certain cancer cell types. However, the molecular details of induction of apoptosis upon Hsp90 inhibition are not understood. We have investigated the effect of Hsp90 inhibition on a non-adherent rat histiocytoma cell line, BC-8, using geldanamycin and 17-Allylamino-17-demethoxygeldanamycin. We show that Hsp90 inhibition induces ER stress, which leads to disruption of mitochondrial homeostasis, leading to apoptosis. Induction of ER stress leads to increased expression of ER chaperones, Grp78 and Grp94, cleavage of caspase-12 and increase in cytoplasmic calcium. We show that calcium and Bax are responsible for the decrease in mitochondrial membrane potential ($\Delta\psi_m$), thereby leading to the release of cytochrome *c* and activation of caspase-9. Moreover, calcium chelator and over-expression of Bcl-2 is able to confer protection against apoptosis upon Hsp90 inhibition. We conclude that inhibition of Hsp90 leads to ER stress-induced mitochondria-mediated apoptosis and that Bax and Ca^{2+} play an important role in mitochondrial damage.

1. Introduction

Heat shock proteins are critical for several cellular functions including protein folding and assembly. The 90 kDa heat shock protein (Hsp90), an ATPase-directed chaperone [1] is one of the most abundant cytosolic molecular chaperones, comprising 1–2% of the total cellular protein under non-stressed conditions. Its contribution to various cellular processes including signal transduction, protein degradation, protein folding, maturation of client proteins [2] and protein trafficking among sub-cellular compartments has been extensively studied [1,3,4]. Most of the client proteins, which require Hsp90 for their conformational maturation, are kinases and signaling molecules; therefore, Hsp90 occupies a unique role in cellular homeostasis. Consequently, Hsp90 has emerged as a promising anticancer target. Earlier studies show that in cancer Hsp90 is not only up-regulated, but its ATPase activity is also increased about 50-fold [2]. The vulnerable ATPase pocket of Hsp90 has been exploited to inhibit its activity. Hsp90 antagonists geldanamycin (GA) and its derivative 17-allylamino-demethoxygeldanamycin (17-AAG) [5–7] compete with ATP at the ATP binding site and inhibit the intrinsic ATPase activity of Hsp90 [7]. Inhibition of Hsp90 by GA and 17-AAG affects several cellular processes and leads to apoptosis [4].

Apoptosis [8] is a cellular self-destruction mechanism involved in a variety of biological events, such as developmental sculpturing, tissue homeostasis and removal of unwanted cells. Disruption of the regulation of apoptosis is associated with several diseases, including cancer. The family of cysteine proteases, popularly known as caspases, is a critical mediator of programmed cell death. Some of these proteins such as caspase-8 mediate signal transduction downstream of death receptors located on the plasma membrane, whereas others such as caspase-9, mediate apoptotic signal after mitochondrial damage. Mitochondria are pivotal to apoptotic signal amplification [9]. However, other organelles, including the endoplasmic reticulum (ER), have also been implicated [10]. ER stress, caused by accumulation of unfolded protein (termed as unfolded protein response or UPR) or by inhibition of ER-Golgi transport, also results in apoptosis [11,12]. Apoptosis caused by excessive ER stress is mediated by caspase-12, which resides on the ER outer-membrane and is cleaved and activated during ER stress [13].

Antiapoptotic and proapoptotic members of the Bcl-2 family also play an important role in ER-mediated apoptosis with some members such as Bcl-2 and Bcl-X_L inhibiting apoptosis and others

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such as Bcl-2 associated X protein (Bax) inducing apoptosis [14]. Majority of Bcl-2 family proteins are anchored in the outer membrane of mitochondria. During apoptosis, several mitochondrial events, including loss of mitochondrial membrane potential ($\Delta\psi_m$) occur. Apart from these events, proteins such as cytochrome *c* and other apoptosis inducing factors are released from the mitochondria into the cytosol. Upon entering the cytosol, cytochrome *c* forms a multiprotein complex with Apaf-1, known as the apoptosome complex that induces and activates caspase-9, which results in activation of caspase-3, the main executioner caspase [15]. Over-expression of Bcl-2 is known to prevent the loss of $\Delta\psi_m$, release of cytochrome *c* and activation of caspase, whereas Bax induces these changes [14,16]. After apoptotic stimuli, Bax forms multimers and translocates to the outer mitochondrial membrane. Translocation of Bax to the mitochondria is associated with the release of cytochrome *c* from the mitochondrial inter-membrane space and loss of mitochondrial membrane potential ($\Delta\psi_m$) [17].

Another important factor, which contributes to ER-mediated apoptosis, is the disruption of Ca^{2+} homeostasis. Studies demonstrate that Bax can modulate cytoplasmic Ca^{2+} and ER Ca^{2+} stores and change mitochondrial matrix Ca^{2+} ($Ca_m{}^{2+}$) contents [18]. Over-expression of Bax is reported to favor the transfer of Ca^{2+} from ER to mitochondria, thus inducing cell death [18]. Ca^{2+}-independent effects of Bax and Bax independent effect of Ca^{2+} on mitochondria have also been explored. Further, mitochondrial membrane integrity is altered by excessive Ca^{2+} uptake. Transient increase in cytosolic Ca^{2+} levels activates $Ca_m{}^{2+}$ uptake and promotes the accumulation of this ion in the mitochondria [19].

The role of Hsp90 in cell proliferation and survival is well documented; however its inhibition has been shown to induce apoptosis through various pathways [20–22]. Despite the increasing number of studies, a molecular detail of the pathway of cell death upon Hsp90 inhibition is not yet understood. Our studies, described here, show that Hsp90 inhibition induces apoptosis through ER stress as evident by increased levels of Grp94 and Grp78, apart from inhibition of proliferation by inducing cell cycle arrest. Moreover we observed a decrease in the levels of major pro-survival signaling molecules such as Akt upon Hsp90 inhibition. Further, ER stress leads to disruption of mitochondrial homeostasis (decreased mitochondrial membrane potential) thereby inducing apoptosis in BC-8, a rat histiocytoma cell line and a monoclonal population of Ak-5 tumor which leads to tumor development in wistar rats when injected sub-cutaneously or intraperitoneally, that we have used in the present study. Importantly, our results suggest that increase in cytoplasmic calcium, and translocation of Bax and calcium to mitochondria are associated with decrease in mitochondrial membrane potential ($\Delta\psi_m$). Such loss in mitochondrial membrane potential leads to release of cytochrome *c* and activation of downstream executioner caspases. Interestingly, chelation of calcium by cytoplasmic calcium chelator BAPTA-AM was able to partially protect cells from apoptosis. Moreover, over-expression of Bcl-2 protected cells from ER stress-induced apoptosis upon Hsp90 inhibition which further corroborates that the induced apoptosis is through alteration in mitochondrial membrane potential. Thus, our results show that inhibition of Hsp90 leads to ER stress induced, mitochondria-mediated apoptosis and that Bax and Ca^{2+} play an important role in mitochondrial damage.

2. Materials and methods

2.1. Cell line and cell culture

The non-adherent rat histiocytoma cell line, BC-8, a single clone of AK-5 tumor [23] was used for the experiments. Unless otherwise specified, cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Invitrogen, California, USA) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Sigma, St. Louis, USA) in an atmosphere of 95% air and 5% CO_2 at 37 °C.

2.2. Transfection of BC-8 cells with Bcl-2

BC-8 cells (2×10^6) were transfected with linearized pMEP4 vector, with and without full-length murine Bcl-2 gene (kindly provided by Dr. Y.A. Hannun) by the electroporation method. The transfected clones were selected with hygromycin B (400 μg ml^{-1}) for 14 days. Single cell clones were obtained by limiting dilution procedure. Clones were screened by northern hybridization and Western blotting, and the positive clones were expanded and used in these studies.

2.3. Drugs and inhibitors

17-AAG and GA were obtained from NIH. Both drugs were dissolved in DMSO (Sigma, USA) and were used at a final concentration of 1 μM in cell culture. Brefeldin A (Calbiochem, San Diego, CA, USA) was dissolved in DMSO and used at a final concentration of 10 μg/ml in cell culture.

2.4. Reagents and antibodies

Antibodies specific to Hsp90, Bcl-2 associated X protein (Bax) and caspase-3 were obtained from Assay Design (Michigan, USA). Antibodies specific to protein kinase B (Akt), phospho-Akt (p-Akt), glucose regulated protein-94 (Grp-94), glucose regulated protein-78 (BiP), caspase-9, Bcl-2, cyclin dependent kinase 4 (Cdk-4) and cleaved form of caspase-9 were obtained from Santa Cruz Biotechnology (Santa Cruz, California, USA); antibodies to actin and active fragment (85 kDa) of poly (ADP-ribose) polymerase (PARP) were obtained from Chemicon International (California, USA); antibodies specific to procaspase-12 and cytochrome *c* were obtained from BD Pharmingen (NJ, USA). Mouse anti-rabbit Alexafluor-488 secondary antibody was obtained from molecular probes (Eugene, OR, USA). Horse radish peroxidase (HRPO)-conjugated anti mouse/rabbit was obtained from Roche (Indianapolis, IN, USA) and HRPO conjugated anti-goat was obtained from Stressgen (Victoria, BC, Canada). Dyes such as $DiOC_6$ (3,3′-dihexyloxacarbocyanine iodide), BAPTA-AM, Fluo-3 AM ester, Rhod-2 AM, mitotracker red and DAPI (4′,6-diamidino-2-phenylindol) were obtained from molecular probes (Eugene, OR, USA). Bovine serum albumin (BSA) and propidium iodide (PI) were obtained from Sigma (St. Louis, USA). Protease inhibitor cocktail and phosphatase inhibitor cocktail were obtained from Calbiochem (San Diego, CA, USA).

2.5. Cell cycle analysis

Cell cycle and sub-G1 distribution were determined by staining DNA with propidium iodide. Briefly, 1×10^6 BC-8 cells were incubated with Hsp90 inhibitors for the specified time. Cells were then washed with phosphate buffer saline (PBS) and fixed in 70% ethanol overnight at 4 °C. Cells were again washed with PBS and then incubated with 60 μg PI (stock-1 mg/ml in PBS) with simultaneous treatment of RNase (Sigma, St. Louis, USA) at 37 °C for 30 min. The percentages of cells in the different phases of the cell cycle or having the sub-G1 DNA content were measured using a Fluorescence Assisted Cell Sorter (FACS calibur, Becton Dickinson, NJ, USA).

2.6. Annexin V-FITC/PI staining

Apoptosis was determined by staining BC-8 cells with annexin V-fluorescein isothiocyanate (FITC) and PI labeling. Briefly, to

quantitate the apoptosis of cells, 1×10^6 cells were incubated with the designated dose of Hsp90 inhibitor and Brefeldin A for the specified time. Cells were washed twice with cold PBS, and suspended in 100 μL of binding buffer (10 mM HEPES/NaOH [pH 7.4], 140 mM NaCl, and 2.5 mM $CaCl_2$). Then 1 μL of annexin V-FITC (Becton Dickinson, NJ, USA) and PI (2.5 μg) were added to these cells and incubated for 30 min. The cells were analyzed using FACS calibur. Viable cells were negative for both PI and annexin V. Non-viable cells, which underwent apoptosis, were positive for PI and positive for annexin V or only positive for annexin V.

2.7. SDS-PAGE and Western blot analysis

Whole cellular protein was extracted by incubating cells in 50 mM Tris–Cl buffer, pH 8.0, containing, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 150 mM sodium chloride, 1× protease inhibitor and 1× phosphatase inhibitor for 30 min on rototorque at 4 °C. The mix was centrifuged at 12,000 rpm for 25 min at 4 °C. The protein in the resulting supernatant was quantified by the Bradford method (Biorad, Hercules, CA, USA) according to the instructions of the manufacturer, diluted 1:4 in protein SDS loading buffer, and heated to 95 °C for 5–10 min. A total of 20 μg of protein was loaded onto 8%, 10% and 12% Tris–HCl SDS-polyacrylamide electrophoresis gels as required, transferred to Hybond C-extra nitrocellulose membrane (Amersham Biosciences, NJ, USA) using wet transfer apparatus (Biorad, Hercules, CA, USA). The membrane was then incubated in blocking solution [5% BSA in Tris buffered saline (TBS) containing 0.1% Tween-20] for 2 h with gentle rocking at room temperature. Primary antibody was diluted (in 3% BSA in TBS) according to the prescribed dilutions by the manufacturer or was standardized by checking various dilutions. The membrane was incubated for 3–4 h at room temperature or overnight at 4 °C in primary antibody. After washing for 10 min in TBS containing 0.1% Tween-20 (TBST) thrice, the membranes were incubated in secondary antibody conjugated with HRPO. After washing for 10 min in TBST thrice, the substrate (BM Chemiluminescence kit, Roche) was added on the membrane and was developed using Kodak X-ray films. Densitometric scans and comparative calculations were done using ImageJ software (CSHL).

2.8. Caspase-3 activation assay

Approximately 3×10^6 cells were incubated with Hsp90 inhibitors for specified time. Cells were washed with PBS twice after incubation and 0.1 mL of lysis buffer (1% Triton X-100, 0.32 M sucrose, 5 mM EDTA, 10 mM Tris–Cl pH 8.0, 2 mM DTT, 1× protease inhibitor) was added. The mix was incubated for 15 min at 4 °C. The mix was centrifuged for 15 min at 14,000 rpm and supernatant was collected. Protein estimation was performed as described above. 50 μg of protein and 100 μM Ac (*N*-acetyl)-DEVD-AMC (7-amino-4-methylcoumarin) (BD Pharmingen, NJ, USA) were incubated in 50 μL assay buffer (100 mM HEPES pH 7.5, 10% sucrose, 0.1% Chaps, 2 mM dithiothreitol, 1× protease inhibitor) for 1 h at 30 °C. Increase in fluorescence was recorded in a Hitachi F4500 fluorescence spectrophotometer (Tokyo, Japan) with the excitation and emission monochromators set at 380 nm and 460 nm, respectively.

2.9. Preparation of cytosolic extracts for Western blot analysis of cytochrome c

Digitonin-based permeabilization technique was used to release cytosol from cells. Cells were suspended in permeabilization buffer (PBS with 250 mM sucrose, 40 mM KCl, 10 mM HEPES and 100 μg/mL digitonin (Sigma)) after incubating cells with Hsp90 inhibitors. Under these conditions >95% cells were found to be permeabilized when stained with 0.2% trypan blue solution. After incubation for 1 min on ice, the cells were centrifuged at 12,000 × *g* for 5 min at 4 °C. This process was repeated for all the time points. Supernatant was collected as cytosol, electrophoresed on 15% SDS-polyacrylamide gel and blotted on Immobilon-P (Millipore Corp., MA, USA) using semi-dry transfer apparatus (Amersham Biosciences, NJ, USA). Western blot was carried out to detect cytochrome *c*.

2.10. Flow cytometric analysis of mitochondrial membrane potential

Mitochondrial membrane potential was monitored using a cell permeable, green-fluorescent, lipophilic dye, $DiOC_6$ (3,3′-dihexyloxacarbocyanine iodide) that is selective for the mitochondria of live cells, when used at low concentrations. Depolarization of mitochondrial membrane potential ($\Delta\psi_m$) results in the loss of $DiOC_6$ from the mitochondria, thereby decreasing intracellular fluorescence. Briefly, 1×10^6 cells were incubated with Hsp90 inhibitors or Brefeldin A for the specified time. Cells were washed once with PBS and were incubated with 20 nM $DiOC_6$ (diluted in incomplete DMEM) for 20 min. For positive control, cells were treated with 50 μM protonophore *m*-chlorophenylhydrazone (CCCP) for 15 min before incubating them with $DiOC_6$. After incubation, cells were washed twice with PBS and the fluorescence was recorded using FACS calibur in FL-1H log channel.

2.11. Live cell imaging and flow cytometric analysis for cytoplasmic calcium

Approximately 1×10^6 cells were treated with Hsp90 inhibitor or Brefeldin A. Cells were washed once with PBS and incubated with 10 μM Fluo-3 AM and 1 mM mitotracker red (diluted in PBS) for 1 h in an incubator at 37 °C and 5% CO_2. Cells were washed thrice with PBS after incubation; flow cytometry (FACS calibur) was carried out. Another group of cells that were treated with Hsp90 inhibitor or Brefeldin A and labeled with Fluo-3 AM and mitotracker were plated in 2-well Labtek chamber cover glass (Nunc, Roskilde, Denmark) for live cell imaging. The cells were scanned and photographed using 63× water objective lens of Carl Zeiss 510-LSM confocal laser scanning microscope (Carl Zeiss, GmbH, Germany). The photographs were analyzed by using AIM (LSMIB) software. The images were further processed using Adobe Photoshop version-7.0.

2.12. Flow cytometric analysis for mitochondrial calcium

Approximately 1×10^6 cells were treated with Hsp90 inhibitor or Brefeldin A. Cells were washed once with PBS and incubated with 5 μM Rhod-2 AM (diluted in incomplete DMEM) for 1 h in an incubator at 37 °C and 5% CO_2. Cells were washed twice with PBS after incubation. Cells were incubated in complete DMEM for 4 h at 37 °C and 5% CO_2 after labeling with Rhod-2 AM. Cells were analyzed for increase in Rhod-2 AM fluorescence using Flow cytometry (FACS calibur).

2.13. Indirect immunofluorescence and confocal microscopy

For immunofluorescence analysis, BC-8 cells growing in T-25 flasks (TPP) were washed with PBS. Cells were incubated for 50 min in 1× mitotracker red diluted in incomplete DMEM to stain the mitochondria. Cells were washed twice with PBS to remove extra mitotracker, fixed and permeabilized for 15 min in 0.05% Triton X-100 + 0.005% Tween-20 in PBS containing 4% formaldehyde. After two washes with PBS, cells were incubated for 1 h with blocking solution (1% BSA in PBS). Rabbit anti-Bax antibody (1:100

dilutions in PBS) was added to the cells for 1 h. After washes with PBS, the bound primary antibody was detected using mouse anti-rabbit Alexafluor-488 antibody (1:400 dilution in PBS) for 1 h. The DNA was stained with DAPI for 2 min before mounting with Vectashield (Vector Laboratories, California, USA). The cells were analyzed and photographed using a Carl Zeiss 510-LSM confocal laser-scanning microscope (Carl Zeiss, GmbH, Germany). The images were further processed using Adobe Photoshop version-7.0.

3. Results

3.1. GA and 17-AAG induce cell cycle arrest and antiproliferative effects in rat histiocytoma cell line, BC-8

These are small-molecule inhibitors of Hsp90 that have antiproliferative effect in a variety of cancer cells; 17-AAG has recently entered clinical trials in patients with solid tumors and lymphoma [24]. To investigate the biological effects of Hsp90 inhibition in rat histiocytoma, we incubated rat histiocytoma cells, BC-8, with 17-AAG (1 μM) and GA (1 μM). Vehicle-treated cells were used as controls. Hsp90 has been known to chaperone a variety of kinases, most of which are involved in cell proliferation. In order to examine the effect of Hsp90 inhibition on cell proliferation signaling in BC-8 cells, we monitored the levels of protein involved in major pro-survival signaling pathway, protein kinase B (Akt). We observed a decrease in the protein levels of Akt1/2 as well as in the phosphorylation levels of Akt1/2 (Fig. 1A). No significant change in the levels of extra-cellular regulated kinase 1/2 (ERK 1/2) was observed; however, phosphorylation of ERK 1/2 was completely inhibited (data not shown). Hsp90 is known to be associated with a variety of proteins that are involved in cell cycle progression. Cells treated with Hsp90 inhibitors were stained with propidium iodide and then subjected to FACS analysis. An increase in subG1 population was observed and a gradual decrease in G1 and G2/M phase was noticed in a time-dependent manner (Fig. 1B). In order to investigate the phase in which cells were arrested, we have monitored the levels of major molecules involved in cell cycle progression and inhibition. Cyclin dependent kinase 4/6 (Cdk4/6) is a pivotal molecule, which is associated with CyclinD1, and is required for G1 to S phase progression. We observed a drastic decrease in the levels of Cdk4 with time (Fig. 1A)

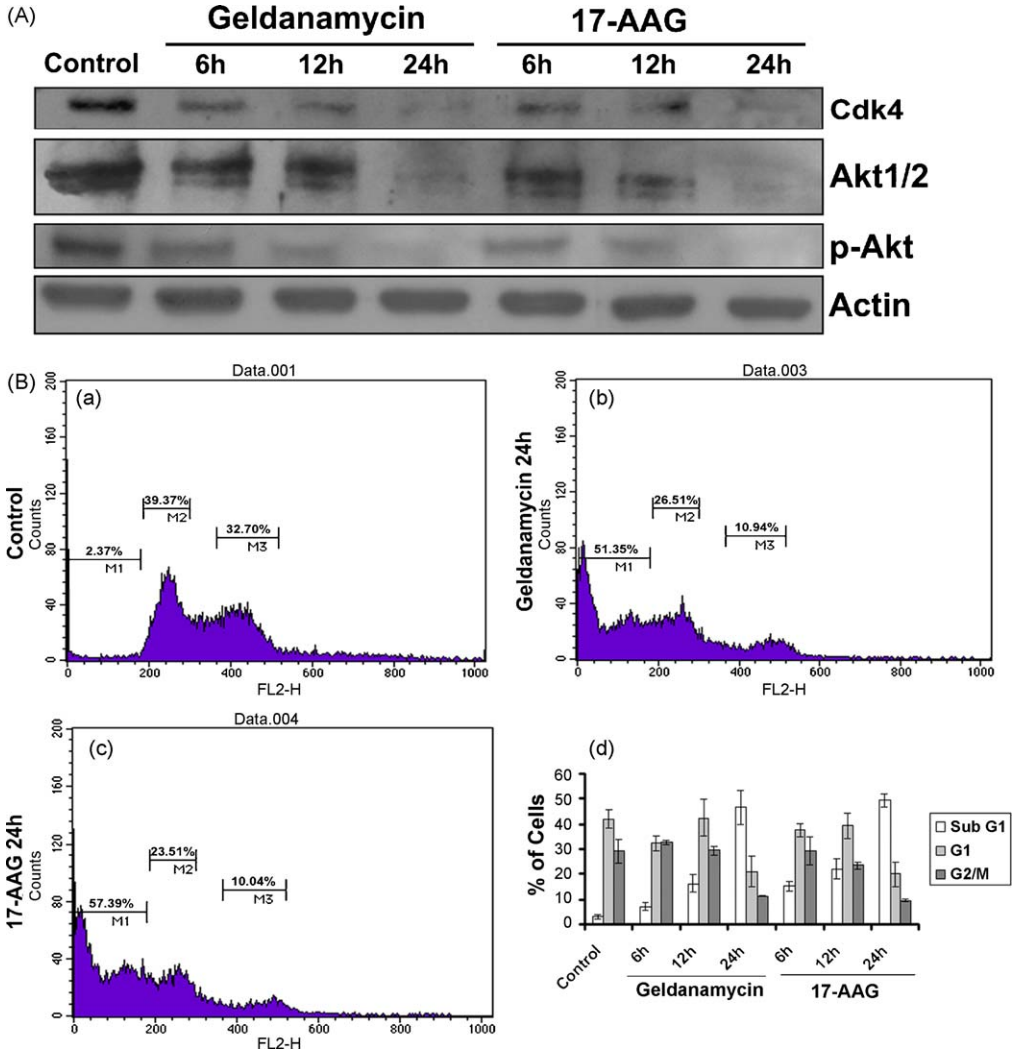

Fig. 1. Hsp90 inhibition by GA and 17-AAG leads to cell cycle arrest and alters the expression levels of major cell survival signaling molecules of BC-8 cells. (A) BC-8 cells were treated with 1 μM concentration of GA and 17-AAG for 6 h, 12 h or 24 h. Whole cell lysates of control and treated samples were subjected to Western blot analysis for Akt1/2, p-Akt1/2 and Cdk 4. The same blot was stripped and used for probing the proteins mentioned above. As a control for protein loading, the blot was probed with anti-actin antibody. The blots are representative of three independent experiments. (B) Cell cycle analysis was done after fixing and staining them with PI (60 μg). Cells were washed and analyzed using FACS calibur. (B) Parts a, b and c represent the FACS analysis of control cells and cells treated with GA and 17-AAG for 24 h, respectively (see Section 2). Part d, graph showing the cells in subG1, G1 and G2/M population upon Hsp90 inhibition by GA and 17-AAG at 6 h, 12 h and 24 h. DMSO treated cells were considered as control. The graph represents the average of at least three independent experiments ± SD.

however the cells were not arrested in the G1 phase of the cell cycle. This data suggests the inhibition of major pro-survival signaling pathways and cell cycle arrest in the subG1 phase upon Hsp90 inhibition: accumulation of cells in subG1 phase clearly indicates cell death.

3.2. Hsp90 inhibition by GA and 17-AAG leads to activation of caspase-3, cleavage of poly (ADP-ribose) polymerase (PARP) and induces apoptosis in BC-8 cells

To investigate the mode of cell death, we have stained the cells after Hsp90 inhibition with annexin V-FITC/propidium iodide. We observed an increase in double positive (annexin V-FITC/PI), staining which indicates cells at later stages of apoptosis. We have also observed single positive stain (annexin V-FITC) which indicates early stages of apoptosis. Both populations of cells increase with time upon Hsp90 inhibition (Fig. 2A). To know whether the effect is persistent, we incubated the cells with Hsp90 inhibitors for longer duration (48 h) and stained with annexin V-FITC/PI. We observed more of double positive (annexin V-FITC/PI) as well as single positive (annexin V-FITC) cells after incubation with GA for 48 h (Fig. 2A). More of single positive (annexin V-FITC) cells were observed after incubation with 17-AAG for 48 h (Fig. 2A). Brefeldin A, a known inducer of ER stress, was used as a positive control. Upon treatment of cells with Brefeldin A, we observed more of double positive population in 24 h. These results clearly show that the mode of cell death is apoptosis. Apoptosis is known to be activated by two different pathways, receptor-mediated (extrinsic) and mitochondria-mediated (intrinsic). To investigate the pathway activated upon Hsp90 inhibition, we monitored the levels of caspase-8, which is known to get cleaved and dimerized in receptor-mediated apoptosis. Activation of caspase-8 was not observed upon Hsp90 inhibition (data not shown), suggesting that apoptosis is not extrinsic.

We observed an increase in the cleaved form of PARP (85 kDa)(Fig. 2B), a general substrate of effector caspases. To confirm the involvement of caspase-3, we performed caspase-3 activation assay. Activity of caspase-3 was found to be maximum at 12 h after Hsp90 inhibition (Fig. 2C), however cleavage of PARP was observed to be maximum at 6 h after Hsp90 inhibition. These observations clearly indicate intrinisic mode of apoptosis and involvement of caspases in apoptosis upon Hsp90 inhibition.

3.3. GA and 17-AAG induce ER stress

Inhibition of Hsp90, as stated above, leads to apoptosis through intrinsic mode. Hsp90 inhibition is also known to cause ER stress. Earlier observations show that GA binds to Grp94 as well as Hsp90 [25], thereby leading to ER stress and up-regulation of ER chaperones [26]. We have, therefore, investigated the Hsp90 inhibition-induced ER stress and its role in the observed apoptosis. Interestingly, we observed an increase in the expression of Grp78/BiP and Grp94, the major ER molecular chaperones, upon Hsp90 inhibition (Fig. 3). The ER stress inducer Brefeldin A, showed similar results. Calpain, a Ca^{2+}-dependent cysteine protease, activation of which is concomitant with the decrease in ER calcium, is known to activate caspase-12. Immunoblot analysis of m-calpain, the isoform which plays the major role in the activation of caspase-12, showed a significant increase upon Hsp90 inhibition in a time-dependent manner (Fig. 3). A similar increase in m-calpain was also observed in cells incubated with Brefeldin A for 24 h (Fig. 3). We also monitored the levels of pro-caspase12, an ER

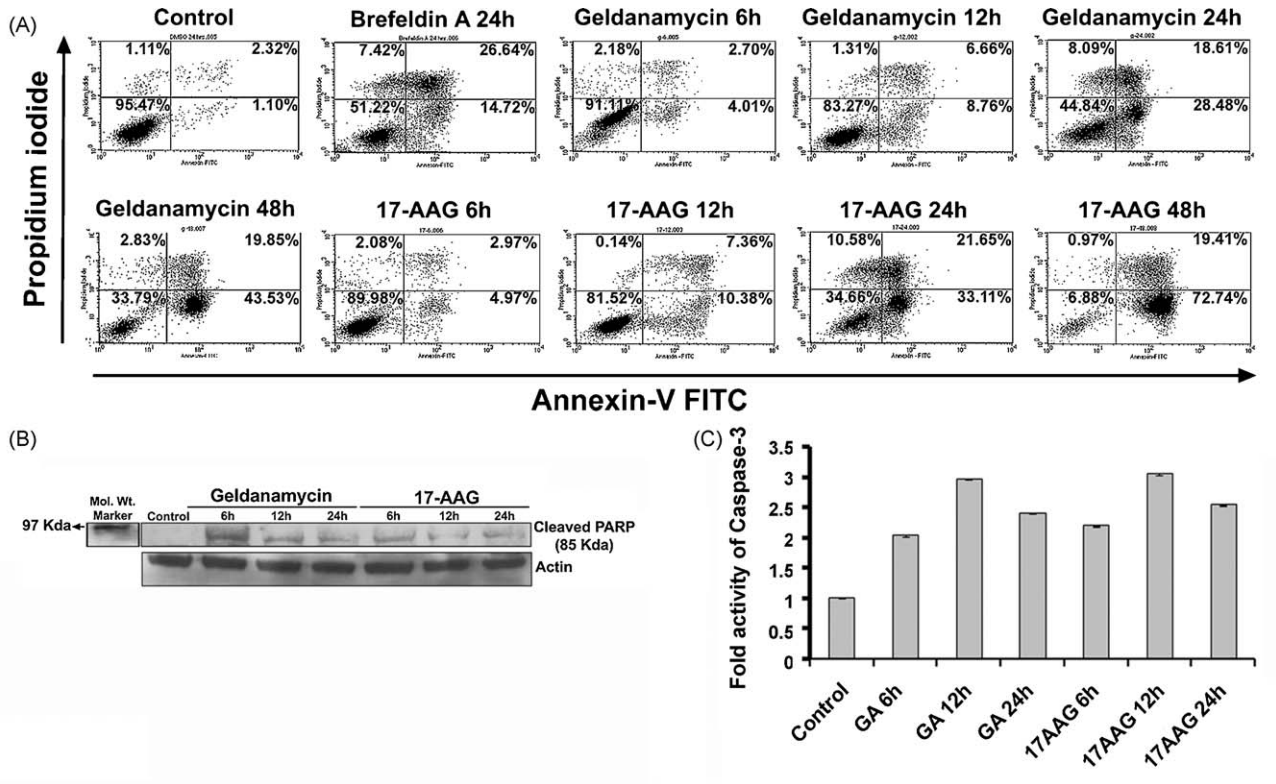

Fig. 2. Hsp90 inhibition induces apoptosis activates caspase-3 and promotes cleavage of Poly (ADP-ribose) polymerase (PARP). BC-8 cells were incubated in GA and 17-AAG for 6 h, 12 h, 24 h or 48 h. (A) Extent of apoptosis was determined by measuring the staining by annexin V-FITC/PI by flow cytometry. Dot blot is the representative of two independent experiments. Brefeldin A treated cells (10 μg/mL) were considered as positive control. *x*-Axis, annexin V; *y*-axis, PI staining. annexin V – positive, PI – negative cells reflect cells in the early stages of apoptosis, whereas annexin V – positive, PI – positive cells reflect dead cells or cells at late stages of apoptosis. (B) Western blot analysis of cleaved form of PARP from whole cell lysates of control and treated cells. Actin was used as a protein loading control. (C) Caspase-3 activity measured by monitoring cleavage of Ac (*N*-acetyl)-DEVD-AMC (7-amino-4-methylcoumarin). Data represents the mean of three independent experiments ± SD.

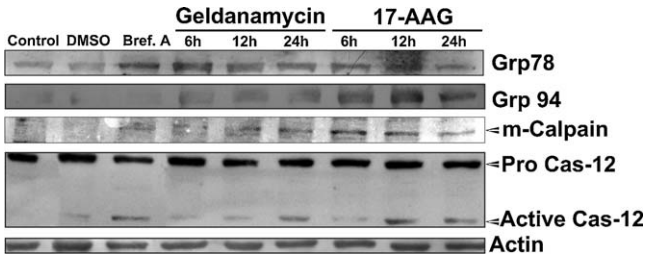

Fig. 3. Inhibition of Hsp90 by GA and 17-AAG induces ER stress in BC-8 cells. BC-8 cells were incubated with Hsp90 inhibitors for 6 h, 12 h or 24 h and with Brefeldin A for 24 h. Whole cell lysates were subjected to Western blot analysis for Grp78, Grp94, m-calpain and caspase-12. Brefeldin A-treated cells were taken as positive control. Untreated cells and DMSO (vehicle) treated cells were considered as control. Actin was used as a control for protein loading. Blots are representative of three independent experiments.

resident caspase which is known to get activated upon ER stress [13]. A significant increase in the cleaved form of caspase-12 was observed upon Hsp90 inhibition in a time-dependent manner as well as with Brefeldin A (Fig. 3).

Previous studies have shown that certain proapoptotic signals associated with ER stress result in depletion of ER calcium store [27] and subsequent release of calcium from ER to the cytosol. We monitored the levels of cytoplasmic calcium with Fluo-3 AM in live cells after Hsp90 inhibition. Increase in cytoplasmic calcium was observed with the increasing time of incubation with Hsp90 inhibitors (Fig. 4B). In order to investigate whether the increase in calcium precedes apoptosis we incubated cells with Hsp90 inhibitors and Brefeldin A and monitored cytoplasmic calcium at early time points. We observed 20–30% increase in cytoplasmic calcium at 9 h after Hsp90 inhibition (Fig. 4A), minimal, only 5–10% of apoptosis was observed at this time. Upon incubation of cells for 18 h with GA and 17AAG (Fig. 4A), we observed 40–50% increase in cytoplasmic calcium which preceded 50–60% apoptosis that was observed after 24 h (Fig. 2A) of Hsp90 inhibition. Cells treated with vehicle (DMSO) showed no increase in the cytoplasmic calcium. This clearly suggests increase in cytoplasmic calcium precedes apoptosis and is not a result of apoptosis.

Increase in cytoplasmic calcium leads to increased uptake of calcium by mitochondria. Uptake of calcium by mitochondria is known to disrupt the mitochondrial homeostasis which leads to

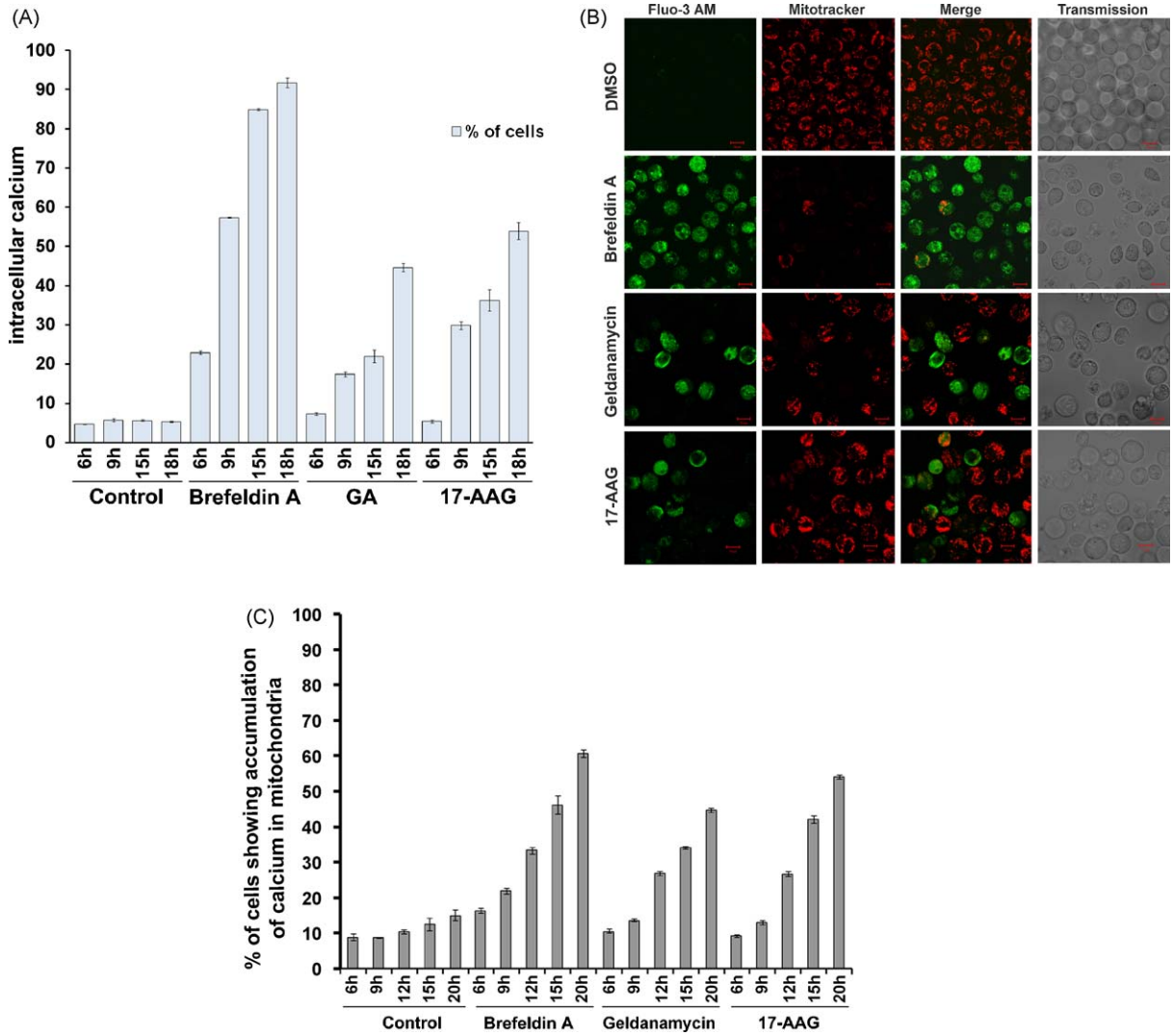

Fig. 4. ER stress induced by Hsp90 inhibition leads to the increase in the levels of cytoplasmic calcium. (A) Cells were treated with GA and 17-AAG for 6 h, 9 h, 15 h and 18 h and levels of cytoplasmic calcium was monitored by FACS. The graph represents the average of three independent experiments ± SD. (B) BC-8 cells were treated with GA and 17-AAG for 18 h. Live cells were stained for calcium with Fluo-3AM (green) and mitotracker red (red) for mitochondria. Cells treated with vehicle and Brefeldin A was considered as control and positive control, respectively. Cells were visualized and scanned using LSM-510 Meta confocal microscope. The phase contrast of the same image is also shown. Scale bars, 10 μm. (C) Cells were treated with GA and 17-AAG for 6 h, 9 h, 12 h, 15 h and 20 h and Brefeldin A as well and levels of mitochondrial calcium was monitored by FACS after staining the cells with Rhod2-AM. The graph represents the average of three independent experiments ± SD.

alteration in membrane potential ($\Delta\psi_m$). Gradual increase in the levels of mitochondrial calcium, monitored by RHOD-2 AM (known to specifically stain mitochondrial calcium), was observed with the increasing time of incubation with Hsp90 inhibitors and Brefeldin A (Fig. 4C). Increase in mitochondrial calcium subsequent to the increase in cytoplasmic calcium clearly suggest uptake of calcium by mitochondria from the cytosol.

3.4. Expression of Bcl-2 associated X proteins (Bax), Bcl-2 and translocation of Bax to mitochondria

Some members of Bcl-2 family of proteins, such as Bcl-2 and Bcl-X inhibit apoptosis while others such as Bax induce apoptosis. Bax is located predominantly in the cytosolic compartment of the cell. After apoptotic stimuli, Bax multimerizes, translocates to the outer mitochondrial membrane. Densitometric scans of the immunoblots of Bax and Bcl-2 reveal a drastic increase in the levels of Bax. However, no significant change in the level of Bcl-2 was observed upon Hsp90 inhibition (Fig. 5A and B). The protein ratio of Bcl-2/Bax (Fig. 5C) decreased indicating an increase in the proapoptotic molecules, which might be responsible for apoptosis. Bcl-X, an antiapoptotic molecule of the Bcl-2 family, showed no change upon Hsp90 inhibition (data not shown). Bax is known to be translocated to mitochondria upon ER stress. Translocation of Bax to mitochondria is associated with the release of cytochrome *c* which forms apoptosome complex and leads to activation of caspases. Immunofluorescence analysis with monoclonal antibody against Bax shows translocation of Bax to mitochondria upon Hsp90 inhibition and with Brefeldin A as well (Fig. 5D). Increase in the levels of Bax protein was also observed with immunofluorescence microscopy (Fig. 5D), which supports our earlier immunoblot studies.

3.5. Mitochondrial membrane permeability transition upon Hsp90 inhibition

Mitochondrion is a pivotal organelle involved in apoptosis apart from its normal function. Translocation of Bax from cytosol to mitochondria is known to create pores in the mitochondrial membrane, thereby leading to disruption of the mitochondrial membrane potential ($\Delta\psi_m$). Uptake of Ca^{2+} by mitochondria from the cytosol or ER also leads to loss of mitochondrial $\Delta\psi_m$. The data discussed above clearly suggests an increase in cytosolic Ca^{2+} and translocation of Bax to mitochondria; both might be independently or interdependently responsible for disturbing the homeostasis of the mitochondrial membrane. We have investigated the change in mitochondrial $\Delta\psi_m$ by labeling the cells with $DiOC_6$, uptake of which is directly proportional to $\Delta\psi_m$, upon Hsp90 inhibition by GA and 17-AAG. We observed a decrease in $\Delta\psi_m$ upon Hsp90 inhibition in a time-dependent manner (Fig. 6A). Both CCCP and Brefeldin A also showed a drastic decrease in $\Delta\psi_m$. However, no noticeable change was observed in cells treated with the vehicle (DMSO). An earlier report showed that the translocation of cyclophilin D to the mitochondrial membrane of HeLa cells was responsible for the decrease in $\Delta\psi_m$ [28]. In order to test if translocation of cyclophilin D into the mitochondrial membrane of BC-8 cells is one of the contributing factors for the observed decrease of $\Delta\psi_m$, we have used cyclosporine A, a molecule which inhibits translocation of cyclophilin D to the mitochondrial membrane. In contrast to the earlier report, we observed a decrease in $\Delta\psi_m$ in cells incubated with both cyclosporine A and Hsp90 inhibitors (data not shown). Thus, the observed decrease in $\Delta\psi_m$ in BC-8 cells can be attributed to inhibition of Hsp90, and not to the translocation of cyclophilin D. Decrease in $\Delta\psi_m$ and consequent membrane-associated changes could lead to the release of cytochrome *c* to the cytosol, which in turn activates caspases leading to apoptosis. We monitored the release of cytochrome *c* from mitochondria to the cytosol upon Hsp90 inhibition. We also investigated the levels of activated form of caspase-9, which is activated by the apoptosome complex during mitochondria-mediated apoptosis (intrinsic apoptosis). We observed an increase in the cytosolic fraction of cytochrome *c* and an increase in the active form of caspase-9 upon Hsp90 inhibition (Fig. 6A–C).

3.6. Cytoplasmic calcium chelator BAPTA-AM and over-expression of Bcl-2 partially protects cells from apoptosis

In order to decipher the potential role of Ca^{2+} in ER stress-induced apoptosis upon Hsp90 inhibition, cells were incubated for 24 h with Hsp90 inhibitors with and without BAPTA-AM (100 nM), a known intracellular calcium chelator which chelates calcium only when internalized by the cell where its acetoxy methyl group gets cleaved. We observed a decrease in apoptosis upon incubation of cells with BAPTA-AM and Hsp90 inhibitors. Cells treated with both GA and BAPTA-AM showed 20–30% reduction in apoptosis as compared to cell treated with GA alone (Fig. 7A). Incubation of cells with both 17-AAG and BAPTA-AM reduced apoptosis by 30–40% as compared to cells treated with 17-AAG alone (Fig. 7A). Interestingly, the cells treated with Brefeldin A and BAPTA-AM also showed reduction in apoptosis. We did not observe any change in the level of apoptosis by incubation of cells with BAPTA-AM alone, showing that the concentration of BAPTA-AM used was not toxic to cells (Fig. 7A). BAPTA-AM showed protection, albeit partial, from apoptosis upon induction of ER stress by Hsp90 inhibition. Partial and not complete protection from apoptosis can be attributed to Bax which might not have been affected upon calcium chelation. In order to study the effect of calcium chelation on Bax, cells were incubated with Hsp90 inhibitors with and without BAPTA-AM for 24 h, and translocation of Bax to mitochondria was checked in the cells undergoing apoptosis. We observed an increase in translocation of Bax to mitochondria upon treatment of cells with Hsp90 inhibitors in the presence of BAPTA-AM in the cells programmed for apoptosis. Interestingly, these cells also showed an increase in the levels of Bax (see supplementary Fig. 1). Neither increase in the expression nor increased translocation of Bax was observed in cells treated with DMSO (control) and BAPTA-AM alone (see supplementary Fig. 1). The data confirms that Bax acts independent of calcium in inducing apoptosis upon Hsp90 inhibition.

Further cells over-expressing Bcl-2 showed a drastic decrease in apoptosis as compared to cells treated with Hsp90 inhibitors alone. Bcl-2 is known to sequester Bax in cytoplasm and hence inhibits its translocation to mitochondria. BC-8 cells over-expressing Bcl-2 showed 80–85% reduction in apoptosis as compared to normal cells when treated with GA, whereas we observed 60–65% reduction in apoptosis as compared to normal BC-8 cells when incubated with 17-AAG (Fig. 7B). We also observed a similar pattern with cells treated with Brefeldin A.

4. Discussion

Hsp90 is an abundant cytosolic molecular chaperone which is involved in maturation and conformational stabilization of proteins, most of which are involved in transducing proliferative and survival signals. Inhibition of Hsp90 has been reported to induce apoptosis in certain cancer cells types [29,30]. Hsp90 is known to be associated with a number of proteins which appear upstream in the survival signaling cascade. Thus inhibiting Hsp90 will lead to inhibition of multiple signaling molecules and hence pathways. Thus there can be multiple mechanisms of cell death by Hsp90 inhibition. For example, Hsp90 inhibition leads to

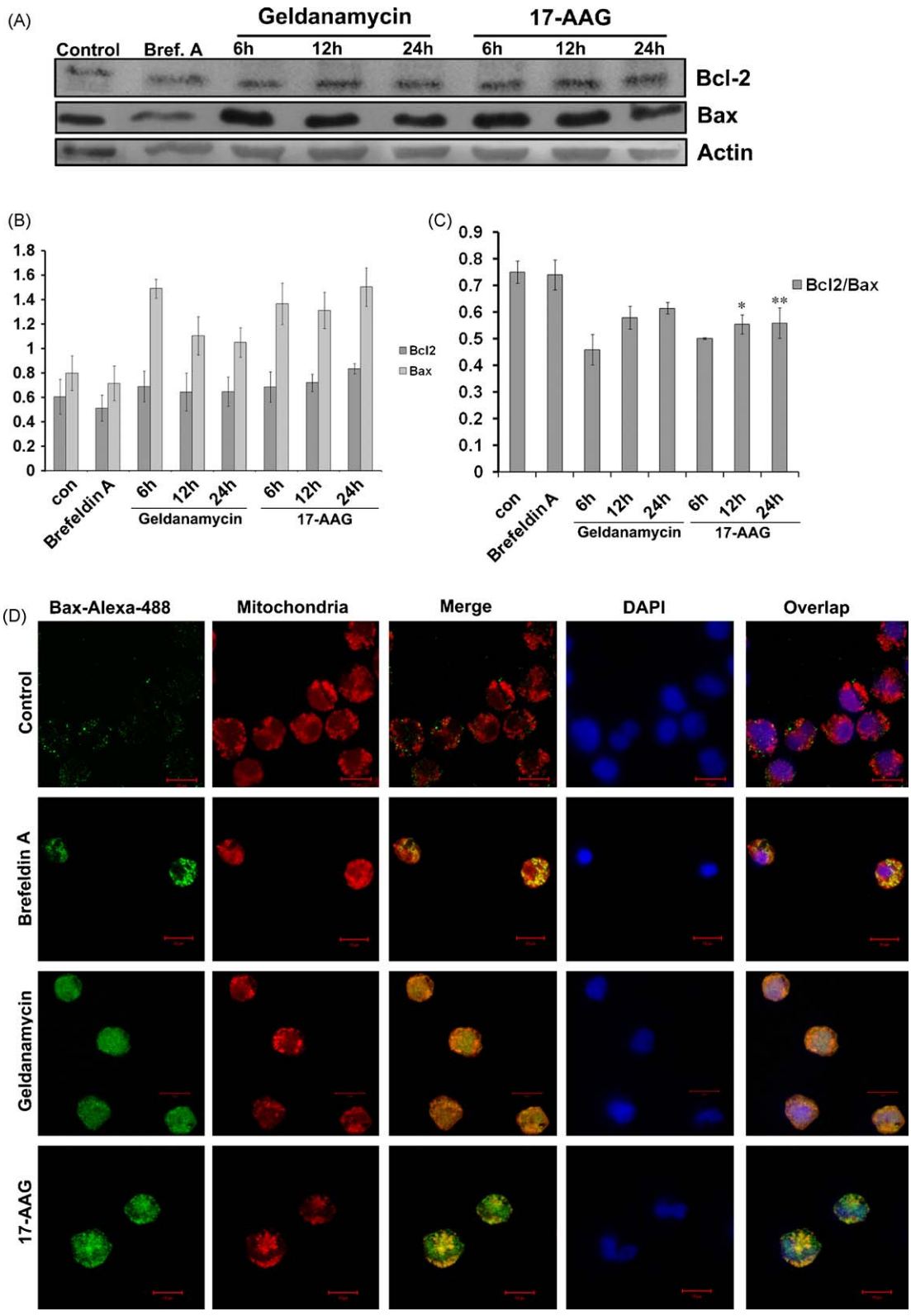

Fig. 5. Expression levels of Bcl-2 associated X protein (Bax), Bcl-2 and translocation of Bax to mitochondria. (A). BC-8 cells were incubated with GA and 17-AAG for 6 h, 12 h or 24 h and with Brefeldin A for 24 h and whole cell lysates were subjected to Western blot analysis for Bax and Bcl-2. As a control for protein loading blot was probed with anti-actin antibody. (B) Bar diagram (graph) representing the average of densitometric scan normalized with actin obtained from three independent experiments ± SD. (C) Graph representing the ratio of Bcl-2/Bax protein obtained from three independent experiments ± SD. Statistical analysis was performed using a Student's *t*-test. The level of significance of the difference with control cells is indicated as follows: $^{*}p < 0.05$, $^{**}p < 0.07$. (D) Cells were incubated with Hsp90 inhibitors or Brefeldin A for 18 h and were stained for mitotracker red (red) and Bax (green). Cells treated with DMSO were considered as control. Live cells were stained for mitochondria by incubating them with mitotracker red and were subsequently fixed and stained for Bax (green). The cells were visualized and scanned under LSM-510 Meta confocal microscope. Merge of mitotracker (red) and Bax (green) and overlap image of DAPI-stained DNA, mitotracker and Bax is also shown. Scale bars, 10 μm.

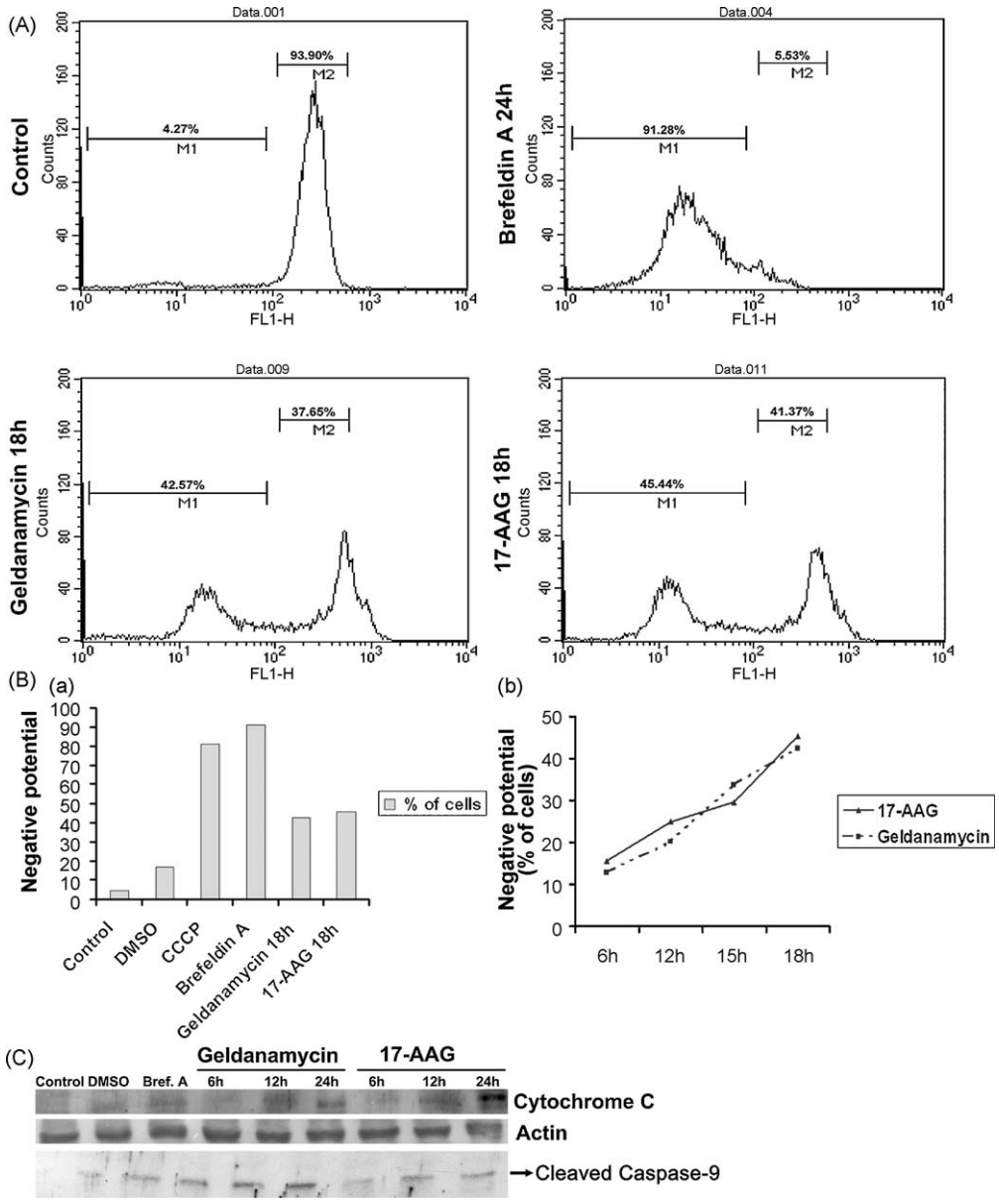

Fig. 6. Change in $\Delta\psi_m$ upon Hsp90 inhibition by GA and 17AAG. BC-8 cells were incubated with GA and 17-AAG for 6 h, 12 h, 15 h, 18 h or 24 h. $\Delta\psi_m$ was measured by staining live cells with $DiOC_6$. CCCP, a protanophore, and Brefeldin A were used as positive controls. Untreated cells and cells treated with DMSO (24 h) were considered as control. After staining with $DiOC_6$, cells were subjected to FACS and mitochondrial membrane potential transition was measured (see materials and methods). (A) Parts a, b, c and d, showing mitochondrial membrane potential transition of control cells, cells treated for 24 h with Brefeldin A, and cells treated for 18 h with GA and 17-AAG, respectively. (B) Part a, bar graph represents the percentage of cells showing decrease in ψ_m after treatment with Hsp90 inhibitors and Brefeldin A. CCCP, a protanophore, is used as an experimental positive control; Part b, the line graph represents the increase in percentage of cells showing decrease in ψ_m as a function of time of incubation with Hsp90 inhibitors. (C) BC-8 cells were incubated with GA and 17-AAG for 6 h, 12 h and 24 h. Cytosolic fraction was isolated by digitonin permeabilization method (see Section 2). Proteins were separated on 15% SDS-PAGE and were transferred to Immobilon-P membrane using a semi-dry transfer apparatus. The membrane was further processed to detect cytochrome *c*, processed capase-9 and actin. The blot is representative of two independent experiments.

proteasomal degradation of Raf-1, an upstream mitogen activated signaling cascade molecule, leading to apoptosis [31].

We have investigated the effect of Hsp90 inhibition on a non-adherent rat histiocytoma cell line, BC-8, using functional inhibitors, GA and 17-AAG. Our data shows that the functional inhibition of Hsp90 leads to generation of ER stress: ER stress was accompanied with the increase in cytosolic calcium which leads to increase in mitochondrial calcium levels. Our data also shows translocation of Bax, a pro-apoptotic molecule, to mitochondria leading to decrease in mitochondrial membrane potential and cytochrome *c* release. The results of our study suggest ER stress induced, mitochondria mediated apoptosis upon Hsp90 inhibition: this process is associated with increase calcium and translocation of Bax.

ER stress, induced by Brefeldin A or tunicamycin is known to regulate cell survival pathway in many cell types. An earlier study has demonstrated that inhibition of Hsp90 by GA leads to an increase in the transcription of ER chaperones [26]. Our data shows induction of ER stress which is demonstrated by the increase in Grp94 and Grp78 (ER chaperones) upon Hsp90 inhibition (Fig. 3). However, activation of the upstream ER stress signaling kinase, p-PERK was not observed (unpublished observation) suggesting the existence of other pathway for their activation. It has been shown that during ER stress, caspase-12 is active and acts as a key mediator of ER stress-mediated apoptosis [32]. Caspase-$12^{-/-}$ mouse embryonic fibroblast and primary cortical neurons have been shown to be resistant to apoptosis induced by ER stress

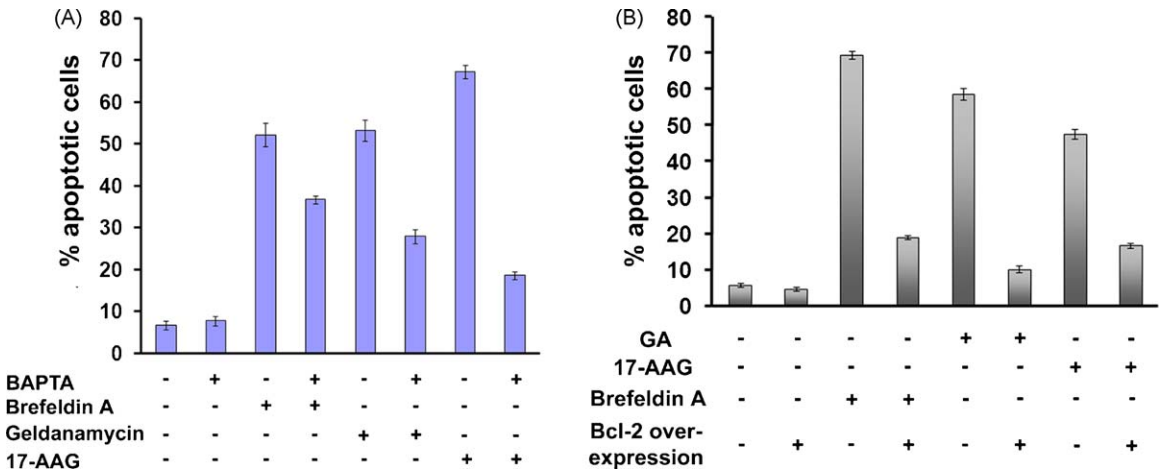

Fig. 7. Chelation of calcium with BAPTA-AM and over-expression of Bcl-2 affects Hsp90 inhibition induced apoptosis. (A) BC-8 cells were treated with GA and 17-AAG with and without BAPTA-AM (100 nM) for 24 h. Cells were washed with PBS and stained with annexin V-FITC/PI and were subjected to FACS. Bar graph shows percentage of apoptotic cells. The graph represents the mean of three independent experiments ± SD. Cells treated with DMSO were considered as control. (B) Over-expression of Bcl-2 reduces apoptosis by Hsp90 inhibition. BC-8 cells over-expressing Bcl-2 and without over-expression was treated with Hsp90 inhibitors and Brefeldin A for 24 h. Cells were washed with PBS and stained with annexin V-FITC/PI and were subjected to FACS. Bar graph shows percentage of apoptotic cells. The graph represents the average of three independent ± SD. Cells treated with DMSO were considered as control.

inducing agents such as Brefeldin A [13]. Our results show that upon Hsp90 inhibition, pro-caspase-12 (M_r 55 kDa) gets cleaved into active caspase-12 (M_r 34 kDa), further supporting that Hsp90 inhibition indeed results in ER stress. Earlier reports suggest that ER stress leads to activation of m-calpain, a calcium dependent event [33,34]. Our calcium measurement studies with Fluo-3AM dye shows that the cytosolic calcium gets increased (Fig. 4A and B) upon Hsp90 inhibition. Increase in cytosolic calcium might be due to release of calcium from ER. The decrease in the ER calcium (and subsequent increase in cytoplasmic calcium) may lead to activation of m-calpain as shown in Fig. 3 [35]. The cytosolic calcium further translocates to mitochondria leading to change in mitochondrial membrane potential.

The balance between anti-apoptotic and pro-apoptotic molecule such as Bcl-2 and Bax, respectively, is necessary for maintaining the homeostasis between cell survival and death. The knock down of Bax is known to confer protection against lethal ER stress induced by tunicamycin [36]. Earlier reports show that stress signals are transmitted from ER to mitochondria, and that the ER stress-induced apoptosis seems to be mediated through mitochondria [32,36,37]. We observed an increase in the expression of Bax and a decrease in Bcl-2/Bax ratio which clearly suggest an increase in the proapoptotic signaling. In addition our data shows that Bax gets translocated to mitochondria due to ER stress generated upon Hsp90 inhibition, which may affect the mitochondrial membrane. Whether the change in the mitochondrial membrane potential is due to calcium/Bax alone or is a synergistic effect needs to be investigated. The migration of Bax is known to induce cytochrome *c* release from mitochondria [16] which leads to activation of caspase-9 and caspase-3. Activation of caspase-3 leads to cleavage of PARP resulting in apoptosis upon Hsp90 inhibition as demonstrated by annexin V-PI staining (Fig. 2). Earlier studies have shown that the level and extent of phosphorylation of Akt, a major pro-survival signaling molecule associated with Hsp90, decrease upon Hsp90 inhibition [38]. This is in agreement with our findings; we observed a decrease in the protein level of Akt as well as a significant decrease in its phosphorylation, upon Hsp90 inhibition (Fig. 1A). We also observed a significant decrease in Cdk4 level and increase in subG1 population of cells (Fig. 1A and B) ultimately leading to apoptotic cell death. Fig. 7A shows that upon incubation of cells with BAPTA-AM, a cytoplasmic calcium chelator leads to decrease in apoptosis suggesting that calcium is involved in induction of apoptosis. Translocation and increased expression of Bax in the presence of calcium chelator demonstrates that both calcium and Bax might be independently involved in the apoptotic process upon Hsp90 inhibition.

Bcl-2 was initially discovered as a proto-oncogene, whose over-expression suppressed apoptosis in a wide range of cell types [27].

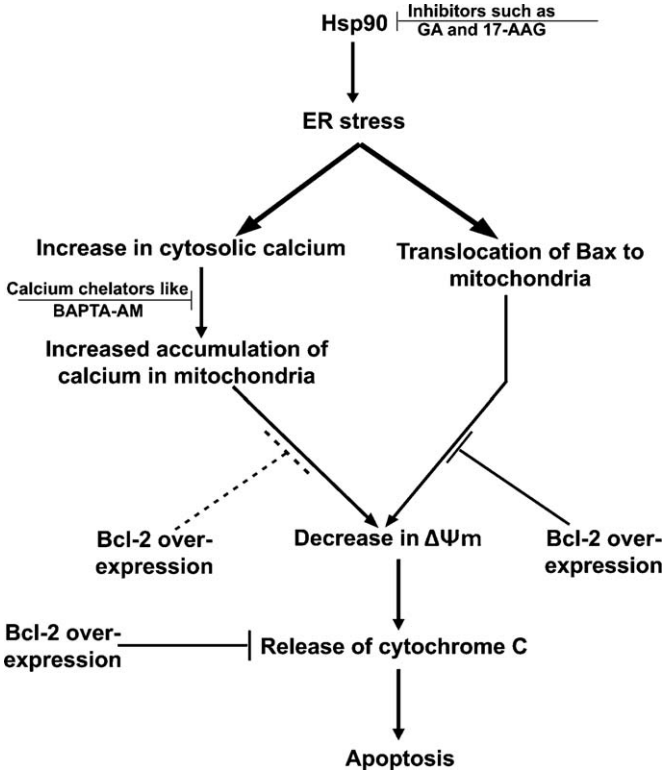

Fig. 8. Schematic description of Hsp90-inhibition-induced apoptosis. Model depicts that Hsp90 inhibition induces ER stress which further leads to increase in cytosolic calcium and translocation of Bax to mitochondria. Increase in cytosolic calcium leads to increased accumulation of calcium in mitochondria. Bax translocation to mitochondria and increased mitochondrial calcium leads to loss in mitochondrial transmembrane potential thereby leading to apoptosis. Chelation of cytoplasmic calcium by BAPTA-AM and over-expression of Bcl-2 leads to decrease in Hsp90 inhibition induced apoptosis by possible mechanisms shown.

Earlier report shows that Bcl-2 over-expression protects cells from mitochondria-mediated apoptosis [14]. Increased expression of Bcl-2 protects cells from apoptosis due to repartitioning of calcium upon withdrawal of interleukine-3 (IL-3) [27]. We observed more than 80% protection from ER stress-induced apoptosis in Bcl-2 over-expressing BC-8 cells as compared to normal BC-8 cells upon Hsp90 inhibition. It is possible that Bcl-2 might be sequestering Bax in the cytoplasm and/or inhibiting the release of cytochrome *c* from mitochondria [14,37,39].

We conclude that two pathways, one through Bax and the other through calcium, exist for apoptosis due to generation of ER stress upon Hsp90 inhibition which may or may not be inter-connected. Fig. 8 schematically summarizes our results. The scheme delineates the apoptotic pathway upon Hsp90 inhibition and also shows where Bcl-2 and calcium might be interfering with apoptosis. Taken together our data provides a mechanistic insight into how inhibition of Hsp90 leads to apoptosis and also establishes a link between mitochondria and ER during ER stress-induced mitochondria-mediated apoptosis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2009.04.001.

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