Geldanamycin, an Inhibitor of Hsp90, Potentiates Cytochrome P4502E1-Mediated Toxicity in HepG2 Cells

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Abstract
Cytochrome P450 2E1 (CYP2E1) potentiates oxidative stress-mediated cell death. Heat shock proteins (Hsps) modulate the stability and function of numerous proteins. We examined the effect of geldanamycin (GA), an inhibitor of Hsp90, on CYP2E1-mediated toxicity in transfected HepG2 cells overexpressing CYP2E1 (E47 cells). Basal expression of CYP2E1 and Hsp90 was higher in E47 cells compared with control C34 cells, which do not express CYP2E1. Treatment with GA resulted in significant toxicity to E47 cells compared with C34 cells. An enhanced loss of E47 cell viability was also observed using two different inhibitors of Hsp90, herbimycin A and radicicol. Treatment of E47 cells with GA caused depletion of glutathione coupled to an increase in reactive oxygen species level and lipid peroxidation. These effects of GA were more pronounced in the E47 than the C34 cells. The antioxidants trolox and N-acetylcysteine prevented the increased reactive oxygen species accumulation and resultant loss of viability. GA caused increased caspase 3 activity and Annexin V staining in E47 cells, suggesting an apoptotic mode of cell death. A decrease in mitochondrial membrane potential was observed in GA-treated HepG2 cells, and mitochondrial permeability transition inhibitors prevented the cytotoxicity of GA. These results suggest that Hsp90 is protective against CYP2E1-dependent oxidant stress and loss of cell viability in HepG2 cells.

Molecular chaperones assist in the proper folding and assembly of proteins, their translocation across a variety of intracellular membranes, and targeting of misfolded proteins for degradation (Hartl, 1996; Hayes and Dice, 1996). These proteins also stabilize unfolded proteins and prevent inappropriate association or aggregation of proteins (Hartl, 1996; Hayes and Dice, 1996). Heat shock proteins (Hsps) are a group of chaperone proteins that promote protein disaggregation by catalyzing the refolding of damaged or denatured proteins and also target unfolded proteins for degradation when cells are subjected to heat shock or other types of stress (Lewis et al., 2000; Pandey et al., 2000; Katschinski et al., 2002). Heat shock protein 90 (Hsp90), a 90-kDa protein, is the most abundant chaperone in the eukaryotic cytosol, and its expression is stimulated one in the eukaryotic cytosol, and its expression is stimulated when cells are exposed to stress like high temperature (Lewis et al., 2000; Pandey et al., 2000; Katschinski et al., 2002).

Geldanamycin (GA), a benzoquinone ansamycin antibiotic and natural product of Streptomyces geldanus that has anti-tumor activity, specifically binds with high affinity to ATP-binding sites of Hsp90 (Lewis et al., 2000; Katschinski et al., 2002). It blocks the ATP-dependent maturation process of Hsp90-dependent complexes in an intermediate state by interfering with the association of Hsp90 with Hsc70 and, finally, the recruitment of the cochaperone p23 that has been reported to stabilize the interaction between Hsp90 and its regulated proteins, thereby inhibiting its chaperone function (Katschinski et al., 2002; Nomura et al., 2004). The binding of GA to Hsp90 leads to the dissociation of its wide variety of substrates, which include transcription factors and signaling kinases from Hsp90 and their destabilization, ubiquination, and subsequent degradation by the proteasome (Lewis et al., 2000; Nomura et al., 2004).}

Cytochrome P450 2E1 (CYP2E1) metabolizes numerous low-molecular weight substrates to their active toxic forms including hormone signaling, cell cycle control, and development, prevents protein aggregation, and mediates the ATP-dependent refolding of heat-denatured proteins under stressed conditions (Lewis et al., 2000; Pandey et al., 2000; Katschinski et al., 2002).

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ABBREVIATIONS: Hsp, Heat shock protein; GA, geldanamycin; ROS, reactive oxygen species; PI, propidium iodide; MEM, minimal essential medium; DMSO, dimethyl sulfoxide; NAC, N-acetylcysteine; MTT, 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide; DCF-DA, 2′,7′-dichlorofluorescin diacetate; PBS, phosphate-buffered saline; GSH, reduced glutathione; Ac-DEVD-pNA, acetyl-Asp-Glu-Val-Asp p-nitroanilide; FITC, fluorescein isothiocyanate; MPT, mitochondrial permeability transition.
and thus plays a role in the susceptibility to toxicity and carcinogenicity of potent toxicants (Guengerich et al., 1991; Gonzalez, 2005). The metabolism of certain substrates and uncoupling of the catalytic cycle by CYP2E1 can result in the production of H₂O₂ and superoxide anion radical (ROS) (Ekstrom and Ingelman-Sundberg, 1989; Cederbaum et al., 2001). Ethanol-inducible CYP2E1 also metabolizes ethanol to acetaldehyde and 1-hydroxyethyl radical, and the role of CYP2E1 in potentiating alcohol-mediated hepatotoxicity is well documented (Morimoto et al., 1993; Song et al., 1996; Lieber, 1997).

The possibility that Hsp90 may play a role or contribute to survival in cells expressing high levels of CYP2E1 has not been evaluated. In the present study, we have used HepG2 cells overexpressing CYP2E1 (E47 cells), which have increased ROS production and lipid peroxidation, and control HepG2 cells, which are devoid of CYP2E1 (C34 cells), to examine the possible protective effects of Hsp90 against CYP2E1-mediated toxicity. Using GA, a specific and potent inhibitor of Hsp90, we demonstrate that inhibition of Hsp90 in E47 cells leads to an increased accumulation of ROS, mitochondrial damage, and toxicity, suggesting that Hsp90 is protective against CYP2E1-dependent oxidant stress and toxicity.

Materials and Methods

Reagents. Geneticin (G418 sulfate) was obtained from Invitrogen (Carlsbad, CA). Other chemicals used, including geldanamycin (C29H40N2O9), were obtained from Sigma-Aldrich (St. Louis, MO). Propidium iodide (PI) and rhodamine 123 (Rh123) were purchased from Molecular Probes (Eugene, OR). All chemicals were of the highest quality commercially available.

Cell Culture and Treatment. This study was performed using C34 HepG2 cells, which do not express CYP2E1, and E47 cells, which are HepG2 cells constitutively expressing human CYP2E1 (Chen and Cederbaum, 1998). Cells were cultured in minimal essential medium (MEM) containing 10% fetal bovine serum and 0.5 mg/ml geneticin, supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin, and 2 mM l-glutamine in a humidified atmosphere in 5% CO₂ at 37°C. Cells were plated at a density of 3 × 10⁴ cells/cm² and maintained in culture medium for 24 h before treatments. GA, radicicol, herbimycin A, and cyclosporin A were dissolved in dimethyl sulfoxide (DMSO), trolox was dissolved in ethanol and N-acetyl cysteine (NAC), and trifluoperazine and fructose were dissolved in water. As controls, the incubation medium was supplemented with ethanol during treatment with trolox to reach the same final solvent concentration, typically 0.1%; this concentration of ethanol had no effect on cellular GSH. The mixture was centrifuged at 13,000 g for 1 min to remove denatured proteins, and GSH was determined by the enzymatic method of Tietze (1969). The GSH content was assayed by following the increase in absorbance at 412 nm for 2 min in a cuvette containing 0.1 M sodium phosphate, 5 mM EDTA buffer, pH 7.5, 0.6 mM 5,5′-dithiobis(2-nitrobenzoic acid), 0.2 mM NADPH, 1 U/ml glutathione reductase, and 10 µl of sample (corresponding to ~100 µg of protein). The increment in absorbance at 412 nm was converted to cellular GSH concentration by using a standard curve with known amounts of GSH.

Caspase 3 Activity Assay. HepG2 cells incubated with 0.5 µM GA or 0.1% DMSO for 36 h were harvested by scraping from the dishes, washed with ice-cold PBS, and resuspended in lysis buffer containing 50 mM Tris, 150 mM NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfate, 1.0% Igepal Ca-630, and 0.5% deoxycholate (pH 8.0) containing protease inhibitors (10 µg/ml aprotonin, 1 mM phenylmethylsulfonyl fluoride, and 10 µg/ml leupeptin) followed by centrifugation at 3000 rpm for 15 min at 4°C, and the supernatant containing the cytosolic and microsomal fractions was used for the study. Protein concentration was determined using the Protein DC-20 Assay Kit (Bio-Rad, Hercules, CA). Sample proteins from HepG2 cell extracts (30 µg) were loaded on a 10% SDS-polyacrylamide gel, electrophoresed, and electroblotted onto 0.4-µm nitrocellulose membranes. Protein immunoblot analysis was carried out using anti-human CYP2E1 polyclonal antibody (1:3000) (kindly provided by Dr. J. M. Lasker, Hackensack Biomedical Research Institute, Hackensack, NJ) and rat anti-Hsp90 monoclonal antibody (1:1000) (StressGen Biotechnologies, Victoria, BC, Canada) as primary antibodies and horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000) (Sigma-Aldrich) and goat anti-rat IgG (1:10,000) (Sigma-Aldrich) as secondary antibodies; respectively. Detection by the chemiluminescence reaction was carried out for 1 min using the ECL kit (GE Healthcare, Little Challont, Buckinghamshire, UK) followed by exposure to Kodak Biomax X-ray film (Eastman Kodak Co., Rochester, NY).

Cytotoxicity Assays. Cells were seeded onto 24-well plates, and after the corresponding treatment, the medium was removed, and cell viability was evaluated by assaying for the ability of functional mitochrondria to catalyze the reduction of thiazolyl blue tetrazolium bromide (MTT) to a formazan salt by mitochondrial dehydrogenases, as described previously (Cao and Cederbaum, 2001).

Measurement of Intracellular Reactive Oxygen Species. Fluorescence spectrophotometry was used to measure production of intracellular levels of ROS with 2’,7’-DCF-DA as the probe as described previously (Bai and Cederbaum, 2003). In brief, HepG2 cells were treated with 0.5 µM GA or 0.1% DMSO for 36 h followed by incubation with 5 µM DCF-DA in MEM for 30 min at 37°C in the dark. The cells were washed in PBS, tryspinized, and resuspended in 3 ml of PBS, and the intensity of fluorescence was immediately read in a fluorescence spectrophotometer (650-10S; Perkin Elmer Life Sciences, Boston, MA) at 503 nm for excitation and at 529 nm for emission. Results were expressed as arbitrary units of fluorescence per 10⁶ cells.

Measurement of Lipid Peroxidation. Lipid peroxidation was measured following a previously described method (Kuyper et al., 1987). In brief, 10⁶ cells were seeded onto six-well plates and incubated for 36 h with 0.5 µM GA or 0.1% DMSO. cis-Parinaric acid was added at a final concentration of 5 µM, and plates were incubated for 30 min at 25°C in the dark. Cells were washed twice with 1× PBS, tryspinized, resuspended in PBS, and the intensity of fluorescence was immediately read at 325 nm for excitation (slit, 3 nm) and 413 nm for emission (slit, 5 nm). Decreased fluorescence reflects increased lipid peroxidation with this probe.

GSH Assay. Cells (1 × 10⁶) were seeded onto 100-mm plates, incubated overnight, and were then treated for 36 h with 0.5 µM GA or 0.1% DMSO. Cells were washed twice with 1× PBS, detached by trypsinization, and treated with 10% trichloroacetic acid to extract cellular GSH. The mixture was centrifuged at 13,000g for 1 min to remove denatured proteins, and GSH was determined by the enzymatic method of Tietze (1969). The GSH content was assayed by following the increase in absorbance at 412 nm for 2 min in a cuvette containing 0.1 M sodium phosphate, 5 µM EDTA buffer, pH 7.5, 0.6 mM 5,5′-dithiobis(2-nitrobenzoic acid), 0.2 mM NADPH, 1 U/ml glutathione reductase, and 10 µl of sample (corresponding to ~100 µg of protein). The increment in absorbance at 412 nm was converted to GSH concentration by using a standard curve with known amounts of GSH.
substrate was calculated from the absorbance values at 405 nm of a standard curve of pNA.

DNA Analysis by Flow Cytometry. The DNA content of the E47 cells was analyzed by flow cytometry to quantify the percentage of apoptotic cells as described previously (Caro and Cederbaum, 2001). Cells (5 x 10^6) were seeded onto six-well plates and incubated with 0.5 μM GA or 0.1% DMSO for 36 h. The cells were then harvested by trypsinization and washed with PBS, followed by centrifugation at 2000 rpm for 10 min. The cell pellet was resuspended in 80% ethanol and stored at 4°C for 24 h. The cells were washed twice with PBS, and the pellet was resuspended in PBS containing 100 μg/ml RNase A, incubated at 37°C for 30 min, stained with PI (50 μg/ml), and analyzed by flow cytometry DNA analysis.

Analysis of Apoptosis by Annexin V Staining. Apoptosis was determined using an apoptosis V-FITC apoptosis detection kit (Oncogene Science, Cambridge, MA). In brief, after treatment with GA, C34 and E47 cells were collected, washed twice in ice-cold PBS, and then resuspended in binding buffer at a density of 1 x 10^6 cells/ml. Fluorescein-labeled Annexin V and PI were added to the cells, followed by an incubation for 15 min before analysis with an FAC Scan (BD Biosciences, San Jose, CA). Ten thousand cells were counted, and Annexin V-FITC-generated signals were detected with an FITC signal detector (BD Biosciences).

Flow Cytometry Analysis of the Mitochondrial Membrane Potential. Changes in the mitochondrial membrane potential were examined by monitoring the cells after staining with Rh123, a membrane-permeable cationic fluorescent dye. HepG2 cells (5 x 10^6) were seeded onto six-well plates, and the cells were incubated with 0.5 μM GA for 24 h. The cells were then incubated with fetal bovine serum-free MEM containing 5 μg/ml Rh123 for 1 h. Cells were harvested by trypsinization, washed with PBS, and resuspended in 1 ml of minimal essential medium. The intensity of fluorescence from Rh123 was analyzed by flow cytometry.

Statistical Analysis. All data are presented as mean ± S.E. and are the results of three experiments. Analysis of variance single factor analysis and Student's t test were used to calculate the statistical significance between C34 and C34 cells and nontreated (DMSO) C34 or E47 cells and GA-treated C34 or E47 cells, respectively. p < 0.05 was considered to be statistically significant.

Results

CYP2E1 and Hsp90 Protein Expression in HepG2 Cells. Immunoblot and densitometric analysis of HepG2 cells showed that C34 cells did not express CYP2E1, and treatment of cells with GA for 36 h did not affect its expression in these cells (Fig. 1A). In contrast, a strong immunoreactive signal of basal CYP2E1 protein was detected in E47 cells. DMSO, which is used as a vehicle for GA, slightly increased the expression of CYP2E1 (1.2-fold). Treating the E47 cells with GA for 36 h increased CYP2E1 slightly further, although the change was insignificant (1.5-fold). A marginal increase (20%) in CYP2E1 catalytic activity as measured by the hydrolysis of 7-methoxy-4-trifluorocumarin was also observed in GA-treated E47 cells (data not shown).

Figure 1B shows the Western blot and densitometric analysis of Hsp90 in HepG2 cells. The constitutive expression of Hsp90 in HepG2 C34 and E47 cells was quite strong, and E47 cells expressed Hsp90 protein at a 2-fold higher level than the C34 cells. Hsp70 levels were similar in the E47 and C34 cells (data not shown). GA did not significantly affect the levels of Hsp90 in either C34 or the E47 cells (data not shown).

Time Course and Dose Response of Cytotoxicity of GA in HepG2 Cells. As shown in Fig. 2A, 0.5 μM GA exhibited a time-dependent toxicity in C34 and E47 cells. There was no toxic effect of GA to C34 or E47 cells during the initial 8 h of treatment. However, at 24 h, GA caused a more significant toxicity in E47 cells compared with C34 cells. At 30 h, only 61% of the E47 cells survived compared with 80% of the viable C34 cells. Further treatment with GA for 36 h lowered the viability of E47 cells to 39%, whereas 79% of the C34 cells were viable during this treatment. At 42 h, the viability of both C34 and E47 cells were decreased to 36 and 20%, respectively.

The toxic effects of various concentrations of GA in C34 and E47 cells are shown in Fig. 2B. At concentrations of 0.1 or 0.2 μM GA, there was an approximately 25% loss of cell viability induced by GA in C34 cells, but only 50 to 55% of the E47 cells were viable at these concentrations. At concentrations of 0.2 to 2 μM GA, caused more than 65% loss of viability of E47 cells; some toxicity was also observed in C34 cells, although it was significantly lower than that in the E47 cells.

Morphological Changes in GA-Treated HepG2 Cells. The morphology of HepG2 cells treated with 0.1% DMSO or 0.5 μM GA for 36 h was recorded by visualizing the cells under a light microscope (Nikon, Kawasaki-ku, Kanagawa, Japan) (data not shown). GA did not cause any significant morphological changes in C34 or E47 cells at 8, 24, or 30 h (data not shown). At 36 h, GA caused substantial morphological changes when added to the E47 cells, since many cells were detached and floated to the top of the culture dish; cells were shrunken and dispersed, and a monolayer was not
formed. Some morphological changes were also observed with the C34 cells treated with GA; however, these changes were more apparent in E47 cells, and much fewer E47 cells could be seen compared with the C34 cells when subjected to the GA treatment for 36 h.

**Cytotoxicity of Radicicol and Herbimycin A in HepG2 Cells.** The dose response of cytotoxicity of radicicol, an inhibitor of Hsp90 that is structurally different from GA, and herbimycin A, another ansamycin antibiotic on C34 and E47 cells, at 24 h was examined (Fig. 3). Radicicol exerted cytotoxic effects on E47 cells at all concentrations used (1–10 μM), and the toxicity was highly significant and greater than that found with the C34 cells; the percentage of viable E47 cells was considerably lower than that seen with C34 cells at each concentration of radicicol (45–65% versus 78–95%) (Fig. 3A). Similar cytotoxic effects were also seen with herbimycin A (Fig. 3B) since the viability for E47 cells was less than that of C34 cells at all concentrations of herbimycin A evaluated (44–73% versus 73–78%) (Fig. 3B). Thus, E47 cells were more sensitive to the toxic effects of three different inhibitors of Hsp90 compared with C34 cells.

**Intracellular ROS Level and Lipid Peroxidation in GA-Treated HepG2 Cells.** The intracellular level of ROS in C34 and E47 cells treated with GA was evaluated by measuring the fluorescence of DCF-DA, a probe which is oxidized by several oxidants such as H₂O₂, superoxide radical, lipid hydroperoxides, lipid peroxyl and alkoxyl radicals, and by cellular peroxidases (Bai and Cederbaum, 2003). E47 cells had a higher basal ROS level than the C34 cells (226 versus 153 arbitrary fluorescence units) (Fig. 4A). Similar cytotoxic effects were also seen with herbimycin A (Fig. 3B) since the viability for E47 cells was less than that of C34 cells at all concentrations of herbimycin A evaluated (44–73% versus 73–78%) (Fig. 3B). Thus, E47 cells were more sensitive to the toxic effects of three different inhibitors of Hep90 compared with C34 cells.

**GSH Content in GA-Treated HepG2 Cells.** GSH is the most abundant nonprotein antioxidant in cells and is important in maintaining proper cellular redox balance and protection of cells against stress and injury (Hall, 1999). As previously reported (Mari and Cederbaum, 2000), E47 cells had 1.5-fold higher GSH content than the C34 cells. This increase was suggested to reflect an attempt to adapt to CYP2E1-dependent oxidant stress. Treatment of C34 cells with GA did not affect GSH level (Fig. 4C). In contrast, when the E47 cells were treated with GA, almost an 11-fold decrease in the GSH content was observed (Fig. 4C).

**Effect of Antioxidants on ROS Levels, Viability, and Morphology in E47 Cells.** The ability of trolox to prevent the morphological changes caused by GA in C34 and E47 cells was evaluated (data not shown). As mentioned above, the morphological changes caused by GA in the C34 and especially the E47 cells included detachment and floating of fatty acid and a chromophore (Kuypers et al., 1987). There was a small increase of approximately 10% in basal lipid peroxidation in E47 cells compared with C34 cells (Fig. 4B). GA had no effect on lipid peroxidation in C34 cells, whereas there was a significant increase in lipid peroxidation in E47 cells, as shown by the 30% decrease in fluorescence of cis-parinaric acid (Fig. 4B). Thus, GA potentiated ROS levels and especially lipid peroxidation to a greater extent in E47 than C34 cells.
the cells, the presence of shrunken and dispersed cells, and a lack of formation of a monolayer. Trolox-treated cells were resistant to these changes as C34 and E47 cells retained their normal shape and formed a monolayer. In addition, after treatment with trolox, the cells were more confluent and adhered to the surface.

To evaluate a role for oxidant stress in the toxicity produced by GA, the E47 cells were treated with GA in the presence of antioxidants trolox and NAC, and ROS levels and cell viability were determined after 36 h. ROS levels doubled in the E47 cells treated with GA (Fig. 5A). Trolox decreased accumulation of ROS in the GA-treated cells by 51%, whereas there was a 37% decrease in the NAC-treated E47 cells (Fig. 5A). Trolox and NAC decreased ROS levels in the presence of GA to the control DMSO levels.

Trolox and NAC were also effective in preventing the cytotoxic effects of GA in E47 cells (Fig. 5B). Only 33% of GA-treated E47 cells remained viable in the absence of any antioxidant, whereas the addition of trolox restored viability to 75%, and NAC increased the percentage of viable E47 cells to 67% (Fig. 5B).

**Mode of Cell Death by GA in HepG2 Cells.** To determine whether caspase 3-mediated apoptosis plays a role in inducing cell death in HepG2 cells because of GA, the activity of caspase 3 in C34 and E47 cells was measured (Fig. 6A). The basal caspase 3 level in C34 and E47 cells was similar. GA did not induce caspase 3 activity in C34 cells; however, a significant increase of 150% in caspase 3 activity was observed in GA-treated E47 cells (Fig. 6A). The percentage of apoptotic E47 cells due to GA treatment was measured by analyzing the DNA distribution of E47 cells by flow cytometry. There was a 12-fold increase in the subG0/G1 fraction (M1 zone, hypodiploid area) in the E47 cells after GA treatment (Fig. 6B).

The mode of cell death, apoptosis, or necrosis produced by GA treatment was also evaluated in C34 and E47 cells by flow cytometry after double staining with Annexin V and PI. When apoptosis occurs, Annexin V is externalized in the plasma membrane, which can be detected by flow cytometry. As shown in Fig. 7, control and viable C34 or E47 cells appear on the Annexin V (−) PI (−) (bottom right quadrant) field. Treatment of C34 and E47 cells with GA resulted in an increase in the fluorescence of Annexin V as evidenced by the enhanced population in the Annexin V (+) PI (−) (bottom left quadrant) field. Treatment of C34 and E47 cells with GA resulted in an increase in the population of cells in the top PI (+) field, which is indicative of cell death by necrosis (Fig. 7). There was an 85% increase in the C34 cell population and a 425% increase in the E47 cell population in the Annexin V (+) PI (−) (bottom right quadrant) field after treatment with GA. Thus, GA treatment of CYP2E1-expressing cells and control HepG2 cells caused mainly an apoptotic mode of cell death.

**Effect of GA Treatment on Mitochondrial Membrane Potential.** The possibility of mitochondrial damage and dysfunction due to GA treatment was evaluated by measuring the mitochondrial membrane potential by flow cytometry after staining with Rh123. Rh123 is a lipophilic cation that is taken up by mitochondria, and its uptake into the mitochondria is proportional to the mitochondrial membrane potential (Lemasters and Nieminen, 1997). C34 and E47 cells exposed to GA for 24 h showed an increase of the number of cells with low rhodamine fluorescence (M1 cells), suggesting a decrease in the mitochondrial membrane potential (Fig. 8). GA caused a 13-fold increase in C34 cells and a 35-fold increase in E47

**Fig. 4.** Effect of GA on intracellular ROS levels, lipid peroxidation, and GSH. A, C34 or E47 cells were treated with 0.1% DMSO or 0.5 μM GA for 36 h, and the levels of intracellular ROS were measured by fluorescence spectrophotometry using 2', 7'-DCF-DA as the probe. All results are expressed as arbitrary units of the fluorescence intensity per 10⁶ cells and reflect mean ± S.E. *, p < 0.05 compared with nontreated (DMSO) C34 or E47 cells. B, lipid peroxidation was evaluated by assaying fluorescence of the probe cis-parinaric acid by fluorescence spectrophotometry. Decreased fluorescence reflects increased lipid peroxidation. All results are expressed as arbitrary units of the fluorescence intensity and reflect mean ± S.E. *, p < 0.05 compared with nontreated (DMSO) C34 or E47 cells. C, C34 or E47 cells were treated with 0.1% DMSO or 0.5 μM GA for 36 h, and the total GSH content of samples was assayed by measuring the rate of 2-nitro-5-thiobenzoic acid production. Results are expressed as mean ± S.E. ***, p < 0.001 compared with nontreated (DMSO) C34 or E47 cells.

**Fig. 5.** Trolox and NAC decrease ROS production in E47 cells and increase E47 cell viability. C34 or E47 cells were pretreated with or without 50 μM trolox or 2 mM NAC and 0.5 μM GA for 36 h. A, the level of intracellular ROS was measured by fluorescence spectrophotometry using 2', 7'-DCF-DA as the probe. The results are expressed as arbitrary units of the fluorescence intensity per 10⁶ cells. B, cell viability was assessed by the MTT assay. Results are expressed as mean ± S.E. ***, p < 0.001 versus E47 + GA. *, p < 0.05 versus E47 + GA.
cells in the M₁ phase. The greater decline in mitochondrial membrane potential produced by GA in E47 cells may contribute to the increased toxicity by GA in E47 cells.

**Effect of Permeability Transition Inhibitors on GA-Induced Cytotoxicity in E47 Cells.** The possible role of the permeability transition in the GA-mediated toxicity in E47 cells was evaluated by studying the effects of the MPT inhibitors cyclosporin A and trifluoperazine plus fructose. Both cyclosporin A and trifluoperazine plus fructose inhibited the toxicity as a result of GA in E47 cells (Fig. 9). Trifluoperazine in combination with fructose completely prevented the cytotoxic effect of GA and restored the viability of the E47 cells to the original level. Cyclosporin A alone produced some toxicity to the E47 cells but increased the viability of the GA-treated E47 cells almost to levels found with cyclosporin A alone (Fig. 9). Higher concentrations of cyclosporin A could not be evaluated because of its increased toxicity. The protection conferred by the MPT inhibitors cyclosporin A and trifluoperazine plus fructose suggests the involvement of the permeability transition pore and of mitochondrial dysfunction in the cytotoxicity of GA in E47 cells.

**Discussion**

Several studies have shown that the expression of Hsps as a cellular response to stress results in protection against the initial insult, increases the process of restoration of the normal cell environment, and promotes resistance to subsequent stress in the cell (Li and Werb, 1982; Lewis et al., 2000; Pandey et al., 2000). This protective role of Hsps could be due to their ability to prevent protein aggregation, promote protein disaggregation by catalyzing the refolding of damaged or denatured proteins, or degrade unfolded proteins (Li and Werb, 1982; Gething and Sambrook, 1992; Lewis et al., 2000; Pandey et al., 2000). Hsp90 is a highly abundant cytosolic protein, and it regulates a variety of biological processes including hormone signaling, cell cycle control, and development (Lewis et al., 2000; Pandey et al., 2000; Katschinski et
GA binds with high affinity to the ATP binding site of Hsp90 family of proteins and prevents its chaperone function (Lewis et al., 2000; Katschinski et al., 2002; Nomura et al., 2004).

CYP2E1 is a major source of ROS and promotes oxidative stress in the cell (Song et al., 1996; Lieber, 1997; Cederbaum et al., 2001; Gonzalez, 2005). The aim of the present study was to examine the role of Hsp90 in modulating the toxic effects of CYP2E1 in HepG2 cells. We first examined the levels of Hsp90 in the C34 and E47 cells. E47 cells expressed approximately a 2-fold higher level of Hsp90 protein than C34 cells in the absence of any treatment. It is interesting to speculate that this overexpression of Hsp90 in E47 cells could be a compensatory mechanism to protect these cells against the toxicity as a result of the high oxidative stress in these cells, thus maintaining the proper cell environment for survival. Such an explanation was previously suggested for the increase in E47 GSH levels (Mari and Cederbaum, 2000; Fig. 4C). Future studies will further characterize this up-regulation of Hsp90 in the E47 cells.

The addition of GA resulted in distinct morphological changes in HepG2 cells—the cells were detached from the
viability was assessed by the MTT assay. Results are expressed as

**Fig. 9.** Inhibitors of the mitochondrial permeability transition, trifluoperazine and cyclosporin A, prevent cell death by GA in E47 cells. E47 cells were treated with 0.1% DMSO or 0.5 μM GA with or without 2.5 μM trifluoperazine + 5 mM fructose or 2 μg/ml cyclosporin A for 36 h, and cell viability was assessed by the MTT assay. Results are expressed as mean ± S.E. *p < 0.05 versus E47 + GA and **p < 0.001 versus E47 + GA.

surface with fewer live cells and with dead cells floating on the top. The CYP2E1-expressing E47 cells showed more severe changes than C34 cells. Inhibition of Hsp90 with GA significantly induced cytotoxicity to E47 cells, reducing cell viability in a dose- and time-dependent manner. Similar cytotoxic effects were observed with the other Hsp90 inhibitors radicicol and herbimycin A. We hypothesize that the higher content of Hsp90 and the antioxidant GSH in E47 cells protect the E47 cells against initial toxicity produced by CYP2E1-derived ROS and thus helps in E47 cell survival. However, lowering these antioxidant protectants, e.g., treatment with buthionine sulfoximine to lower GSH levels (Wu and Cederbaum, 2001) or with GA to inhibit Hsp90 function or other toxic agents, e.g., iron, arachidonic acid, and acetaminophen (Caro and Cederbaum, 2004), overwhelm the protection conferred by the antioxidants and Hsp90.

GA significantly increased the ROS levels in both C34 and E47 cells; however, the increase in ROS in E47 cells was of a greater magnitude. GA did not affect lipid peroxidation in C34 cells but caused a significant increase in E47 cells. GA also did not affect the GSH content in C34 cells but caused a significant depletion of this antioxidant in E47 cells. These results suggest that oxidative stress plays a role in the enhanced toxicity by GA to the E47 cells. Trolox, a potent antioxidant, was effective in reversing the morphological changes in HepG2 cells as a result of GA and restoring the normal structure of both C34 and E47 cells. Trolox and NAC also decreased the level of ROS and significantly restored the viability of E47 cells. These results support the concept that Hsp90 inhibition by GA creates an environment of oxidative stress in the HepG2 cells, especially the E47 cells, and antioxidants are effective in restoring the normal cellular environment.

These studies suggest that Hsp90 protects HepG2 cells against CYP2E1-mediated ROS production and its consequent toxicity. The redox-active properties of GA, which contains a quinone group, are well known, and its cytotoxic effect at micromolar concentrations has been attributed to free radical generation (Billecke et al., 2002; Sreedhar et al., 2003). High concentrations of GA (17–20 μM) have been shown to produce maximal amounts of ROS, whereas ROS production at concentrations less than 5 μM GA is much lower and not significant (Billecke et al., 2002; Dikalov et al., 2002). Indeed, under our conditions, 0.5 μM GA only slightly increased ROS levels in C34 cells and did not increase lipid peroxidation or cause decreases in cellular GSH levels in the C34 cells. Furthermore, other inhibitors of Hsp90, radicicol and herbimycin A, which do not produce ROS, also yielded similar results, as did GA. This suggests that the elevated cytotoxicity found in the presence of GA in E47 cells is not due primarily to ROS production by GA itself but rather reflects an increased state of oxidative stress as a result of Hsp90 inhibition by GA. A recent study also showed that GA significantly increased cell death in retinal pigment epithelial cells treated with the lipid peroxidation product 4-hydroxynonenal, thus suggesting the protective role of Hsp90 in oxidative stress (Kaarniranta et al., 2005).

Caspase 3 activity was not changed significantly in C34 cells upon treatment with GA, but it was elevated significantly in GA-treated E47 cells. Since caspase 3 activity was higher in E47 cells, we investigated whether treatment with GA resulted in apoptosis as assessed by the percentage of Annexin V-stained cells. GA treatment caused an almost 2.0-fold increase in Annexin V positive cells in C34 cells while the number of apoptotic E47 cells increased nearly 5.0-fold. This observation was also supported by the DNA fragmentation analysis of control and GA-treated E47 cells, indicating an increased percentage of E47 cells in the M1 phase after treatment of these cells with GA for 36 h.

Caspases, a family of cysteine proteases with aspartate specificity, exist as inactive precursors in proliferating cells, and when activated, they proteolytically cleave a wide variety of cellular proteins that cause morphological changes and the degradation of chromosomal DNA, leading to apoptosis (Nicholson and Thornberry, 1997). A recent study demonstrated that Hsp90 inhibition by GA in human glioma cells leads to the activation of caspase 3 (Nomura et al., 2004). Furthermore, Hsp90 has been shown to function downstream of caspase 8 but upstream of caspase 3 in a study using NIH3T3 mouse fibroblasts (Zhao and Wang, 2004). Several studies have also reported on the antiapoptotic role of Hsp90 in various in vivo and in vitro models (Lewis et al., 2000; Pandey et al., 2005; Xanthoudakis and Nicholson, 2000; Beere and Green, 2001; Vanden Berghe et al., 2003); our study reports on the antiapoptotic and protective effect of Hsp90 against CYP2E1-dependent toxicity.

Mitochondrial damage and dysfunction is implicated as being important in the overall pathway for apoptosis (Susin et al., 1998). It is also considered to be an important mechanism for CYP2E1-dependent cytotoxicity (Wu and Cederbaum, 2002). There was a decline in mitochondrial membrane potential produced by GA, suggesting that GA causes mitochondrial damage in HepG2 cells. The decline in mitochondrial membrane potential by GA was much greater in E47 cells than in C34 cells. These results suggest that inhi-
bition of Hsp90 causes loss of mitochondrial membrane potential, which may augment CYP2E1-dependent toxicity. The MPT is a regulated Ca\(^{2+}\)-dependent increase in the permeability of the mitochondrial membrane, which causes decreased mitochondrial membrane potential, mitochondrial swelling, and rupture of the outer membrane (Nakagawa et al., 2005). Cyclosporin A, a specific inhibitor of cyclophilin family activity, and the combination of trifluoperazine and fructose have been shown to block the MPT (Brooke et al., 1989; Rauen et al., 2003). We examined the effects of these MPT inhibitors on GA-induced cytotoxicity in the E47 cells and found that cyclosporin A and trifluoperazine plus fructose prevented the toxicity of GA. These results support the previous observations that Hsp90 inhibition by GA causes mitochondrial dysfunction, which can lead to cell death.

In summary, the basal expression of Hsp90 was higher in E47 cells, which may reflect a metabolic adaptation to CYP2E1-generated oxidant stress. Inhibition of Hsp90 by GA in E47 cells caused distinct changes in morphology and a decrease in the number of viable cells. This decrease in viability was also found with other inhibitors of Hsp90 by radiocil and herbinycin A. The addition of GA to E47 cells caused increased ROS accumulation, lipid peroxidation, and depletion of GSH. The oxidative stress created as a result of the loss of Hsp90's protective functions was ameliorated by the antioxidants trolox and NAC, which increased the viability of the E47 cells. Caspase 3 activity and apoptosis were increased in the E47 cells treated with GA. Enhanced damage to mitochondria was evident in GA-treated E47 cells, and MPT inhibitors prevented the cytotoxicity of GA. These results suggest that Hsp90 is protective against CYP2E1-dependent toxicity in HepG2 cells by lowering oxidant stress and helping to maintain mitochondrial function.

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References


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