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Geldanamycin, an inhibitor of Hsp90 increases Cytochrome P450 2E1 mediated toxicity in HepG2 cells through sustained activation of the p38MAPK pathway

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Abstract

Cytochrome P450 2E1 (CYP2E1) can mediate reactive oxygen species (ROS) induced cell death through its catalytic processes. Heat shock protein 90 (Hsp90) is an important molecular chaperone which is essential for cellular integrity. We previously showed that inhibition of Hsp90 with Geldanamycin (GA), an inhibitor of Hsp90 increased CYP2E1 mediated toxicity in CYP2E1 over-expressing HepG2 cells (E47 cells) but not in C34-HepG2 cells devoid of CYP2E1 expression. The aim of the present study was to test the hypothesis that the potentiation of CYP2E1 toxicity in E47 cells with GA may involve changes in mitogen activated protein kinase signal transduction pathways. GA was toxic to E47 cells and SB203580, an inhibitor of p38 MAPK prevented this decrease in viability. The protective effects of SB203580 were effective only when SB203580 was added before GA treatment. GA activated p38 MAPK in E47 cells and this activation was an early and a sustained event. GA elevated ROS levels and lipid peroxidation and lowered GSH levels in E47 cells and these changes were blunted or prevented by treatment with SB203580. Apoptosis was increased by GA and prevented by pretreatment with SB203580. The loss in mitochondrial membrane potential in E47 cells after GA treatment was also decreased significantly with SB203580 treatment. The activity and expression of CYP2E1 and Hsp90 levels were not altered by SB203580. In conclusion, the inhibition of Hsp90 with GA increases the toxicity of CYP2E1 in HepG2 cells through an early and sustained activation of the p38 MAPK pathway.

Keywords

CYP2E1; Hsp90; Geldanamycin; p38 MAPK; SB203580

INTRODUCTION

Molecular chaperones are proteins involved in essential housekeeping functions, such as protein folding, transport of proteins, prevention of aggregation of proteins and regulation of protein turnover [1-3]. Chaperones like heat shock proteins (Hsps) which are induced under stress conditions such as high temperature, hypoxia or other changes in the cellular environment maintain the proper folding and conformation of proteins and help in the degradation of

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damaged proteins, thus maintaining the cellular integrity [1,4]. Heat shock protein 90 (Hsp90), a 90 KDa protein which constitutes 1-2% of the total protein in the cell is ubiquitously expressed under normal physiological conditions [4,5]. Hsp90 is mainly localized in the cytoplasm and the two major cytoplasmic Hsp90 isoforms expressed in human cells, Hsp90 α and Hsp90 β , share 78% homology [4,6]. Hsp90 contributes to the folding and degradation of various cellular proteins and the modulation of the activity of a vast number of client proteins that are involved in cell survival and death pathways [6-8]. Its role in maintaining the normal protein folding and prevention of misfolding and aggregation indicates that Hsp90 is essential for cell survival [6-8]. The chaperone functions of Hsp90 are ATP-dependent conformational cycling reactions. Geldanamycin (GA), a benzoquinone ansamycin and an anti-tumour drug isolated from *Streptomyces hygroscopicus* directly binds to the N-terminal ATP binding site of Hsp90 and inhibits the formation of a complex of Hsp90 with its client protein and its resultant chaperone functions [9-11].

Signal transduction pathways involving specific protein kinases play an important role in mediating various cellular functions. Extracellular stimuli such as stress activate the mitogen-activated protein kinase (MAPK) signaling cascade [12]. The three major types of mammalian MAP kinases include extracellular signal-regulated kinase (ERK1/2), p38 (p38- α , β , γ , and δ) and c-Jun amino-terminal kinases (JNK1, 2, 3). Growth factors and phorbol esters stimulate ERK1/2 while stress stimuli activate JNK and p38 kinases. The multiple MAPKs present in the eukaryotic cells regulate diverse cellular processes which include cell development, survival and death.

The large clientele of Hsp90 substrates include proteins involved in signal transduction pathways and Hsp90 has been shown to bind to tyrosine kinases like Src or insulin receptors or to serine-threonine kinases like Akt and stabilize these proteins prior to signal transduction [13-15]. The anti-tumour activity due to the inhibition of Hsp90 by GA has been shown to affect the activities of proto-oncogene kinases such as ErbB2, EGF, v-Src, Raf-1 and Cdk4 [16].

CYP2E1 which is induced by alcohol metabolizes the oxidation of ethanol and generates reactive oxygen species (ROS) [17,18]. The resultant oxidative stress due to CYP2E1 induction is considered to play a role in alcohol liver damage [19]. The metabolism of several pro-carcinogenic substrates to their active toxic forms is also catalysed by CYP2E1 [20]. Previous studies have indicated a possible role for Hsp90 in the degradation of CYP2E1 [21]. We recently reported that GA, an inhibitor of Hsp90 increases cytotoxicity and promotes ROS accumulation and lipid peroxidation in HepG2 cells over-expressing CYP2E1 (E47 cells) [22]. In the current study, we present data showing that the inhibition of Hsp90 with GA in E47 cells results in the activation of the p38 MAPK pathway, and that the inhibition of this pathway with SB203580-the prototypic inhibitor of p38 MAPK decreases the toxicity of GA in these cells.

MATERIALS AND METHODS

Reagents

SB203580 and Geneticin (G418 Sulfate) were obtained from Calbiochem Inc. and Invitrogen respectively. Geldanamycin and other chemicals used were obtained from Sigma-Aldrich. Propidium iodide (PI) and rhodamine 123 (Rh123) were purchased from Molecular Probes. All chemicals were of the highest quality commercially available.

Cell culture and treatment

C34-HepG2 cells which do not express CYP2E1 and E47-HepG2 cells constitutively expressing human CYP2E1 were used for the study [23]. HepG2 cells were cultured in minimal essential medium (MEM) containing 10% fetal bovine serum (FBS) and 0.5 mg/ml geneticin, supplemented with 100 U/ml penicillin and 100 g/ml streptomycin, and 2 mM L-glutamine in a humidified atmosphere in 5% CO₂ at 37 °C. Cells were plated at a density of 3×10^4 cells/cm² and maintained in culture medium for 24 h before treatments. GA, SB203580, SP600125, LY294002 and PD98059 were dissolved in dimethyl sulfoxide (DMSO). Expression of CYP2E1 in the HepG2 cells was validated by Western blot. DMSO was added to controls at a final concentration of 0.1% which was found to be non-toxic to the C34 or E47 cells.

Western Blotting

Cell samples for Western blotting were prepared by lysing the HepG2 cells in a buffer containing 50 mM Tris, 150 mM NaCl, 0.02% Na azide, 0.1% sodium dodecyl sulfate, 1.0% Igepal Ca-630, and 0.5% deoxycholate (pH 8.0) containing protease inhibitors (10 g/mL aprotinin, 1 mM phenylmethanesulfonyl fluoride and 10 g/mL leupeptin) followed by centrifugation at 3000 rpm for 15 minutes 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). Sample proteins from HepG2 cell extracts (30-80 g) were loaded on an 8% SDS-PAGE for CYP2E1 and Hsp90 and 12% SDS-PAGE for p38MAPK and pp38MAPK and electroblotted onto 0.4 m nitrocellulose membranes. Protein immunoblot analysis was carried out using either anti-human CYP2E1 polyclonal antibody (1:3000) (kindly provided by Dr. J. M. Lasker, Hackensack Biomedical Research Institute, NJ), or rat anti-Hsp90 monoclonal antibody (1:1000) (Stressgen Biotechnologies), or mouse anti-p38 and pp38 monoclonal antibodies (1:1000) (Cell Signaling Technology, Inc.) as primary antibodies and either horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000) (Sigma-Aldrich) or goat anti-rat IgG (1:10,000) (Sigma-Aldrich), or goat anti-mouse IgG (1:2000) (Sigma-Aldrich) as secondary antibodies, respectively. Detection by the chemiluminescence reaction was carried out for 1 min using the ECL kit (Amersham Biosciences) followed by exposure to Kodak Biomax x-ray film (Eastman Kodak Co.).

CYP2E1 catalytic activity

CYP2E1 enzymatic activity was determined by measuring the fluorescence generated from the hydrolysis of 7-methoxy-4-trifluorocoumarin (MFC) at 409/530 nm using 1×10^6 HepG2 cells/mL of Eagle's minimal essential medium without phenol red and fetal bovine serum, incubated with 5 M MFC in acetonitrile at 37°C for 2h [24].

Cell viability Assays

The viability of untreated or 0.5M GA- or 10M SB203580- or SB203580 plus GA treated C34 and E47 cells were assayed using three different methods. HepG2 cells were seeded onto 24-well plates and after treatment with various additions for 36h, cell viability was evaluated by assaying for the reduction of thiazolyl blue tetrazolium bromide (MTT) to a formazan salt, as previously described [25]. A lactate dehydrogenase (LDH) leakage assay was also used to measure cell cytotoxicity. HepG2 cells were washed two times with PBS and resuspended in serum-free MEM without phenol red. The culture medium was collected and the cells were lysed with 1% (v/v) Triton X-100 in PBS for 1 h at 37°C; lysates were collected and stored at 4°C. The activities of LDH in the medium (LDH_{out}) and LDH in the cells (LDH_{in}) were measured using the cytotoxicity detection kit (Roche Diagnostics GmbH) according to the manufacturer's instructions. The cytotoxicity was expressed as percentage LDH release [$100\% \times \text{LDH}_{\text{out}} / (\text{LDH}_{\text{out}} + \text{LDH}_{\text{in}})$]. To assay for trypan blue exclusion, 1×10^4 untreated or GA- or SB203580- or SB203580- and GA- treated cells were seeded onto 24-well culture plates

and incubated for 36 h with MEM containing 10% FBS. After this incubation period, a 0.1-ml solution of 0.05% trypan blue in 0.81% NaCl, 0.06% dibasic potassium phosphate was added to each well. After 5-10 min of incubation, the numbers of viable cells excluding or staining positively (dead cells) for uptake of trypan blue were counted under a light microscope.

Measurement of intracellular Reactive Oxygen Species, Lipid Peroxidation and GSH

Intracellular levels of ROS were measured with 2', 7'-DCF-DA as the probe using fluorescence spectrophotometry as described previously [26]. Briefly, C34 and E47 cells were treated with 0.5M GA or 10M SB203580 or 10M SB203580 plus 0.5M GA or 0.1% DMSO for 36 h, followed by incubation with 5 M DCF-DA in MEM for 30 min at 37 °C in the dark. The cells were washed in PBS, trypsinized and resuspended in 3 ml of PBS and the intensity of fluorescence was immediately read in a fluorescence spectrophotometer (Perkin Elmer Life Sciences) at 503 nm for excitation and at 529 nm for emission. Results were expressed as arbitrary units of fluorescence per 10^6 cells.

Lipid peroxidation was measured using a previously described method [27]. Cell samples containing 0.3 mg of protein were incubated with 0.4 ml of TCA-TBA-HCl solution (15% w/v trichloroacetic acid, 0.375% w/v thiobarbituric acid, 0.25N hydrochloric acid containing 0.1mM butylated hydroxytoluene (BHT)) in a boiling water bath for 15 min and cooled subsequently in an ice bath. The supernatant obtained after centrifugation of the samples at 1000g for 10 min was used to measure the formation of thiobarbituric acid-reactive components as detected at 535 nm, using an extinction coefficient of 1.56×10^5 /M/cm to calculate malondialdehyde equivalents.

To assay GSH, C34 and E47 cells (1×10^6) were seeded onto 100-mm plates and incubated overnight and were then treated for 36 hours with 0.5 M GA or 10 M SB203580 or 10 M SB203580 plus 0.5 M GA or 0.1% DMSO. After washing the cells twice with $1 \times$ PBS and detachment with trypsinization, the cells were treated with 10% trichloroacetic acid to extract cellular GSH, which was assayed according to the method of Tietze [28]. The reaction mixture containing the cell-extract was centrifuged at 13,000g for 1 min to remove denatured proteins and the GSH content was assayed by measuring the increase in absorbance at 412 nm for 2 min in a cuvette containing 0.1 M sodium phosphate, 5 M 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 l of sample (~100 g of protein). The increase in absorbance at 412 nm was used to measure the GSH concentration by comparison to a standard curve with known amounts of GSH.

Analysis of Apoptosis by Annexin V Staining and DNA Analysis

The annexinV-FITC apoptosis detection kit (Oncogene Science) was used to measure apoptosis. GA- or SB203580- or SB203580 plus GA- treated C34 and E47 cells were harvested, washed twice in cold PBS, and then resuspended in binding buffer at a density of 1×10^5 cells/ml. Fluorescein-labeled Annexin V and PI were added to the cells, and the cells were incubated for 15 min before analysis with a FAC Scan. The Annexin V-FITC generated signals from ten thousand cells were detected with a FITC signal detector.

The DNA content of the HepG2 cells was analyzed by flow cytometry to quantify the percentage of apoptotic cells as described previously [25]. 5×10^5 cells treated with GA- or SB203580- or SB203580 plus GA- were 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 g/ml RNase A, incubated at 37°C for 30 min, stained with PI (50 g/ml), and analyzed by flow cytometry DNA analysis.

Flow cytometry analysis of the mitochondrial membrane potential

Rhodamine 123 (Rh123), a membrane-permeable cationic fluorescent dye was used to measure the changes in the mitochondrial membrane potential. HepG2 cells (5×10^5) were seeded onto 6-well plates and the cells were incubated with 0.5 M GA or 10 M SB203580 or 10 M SB203580 plus 0.5 M GA for 36h. The cells were then incubated with fetal bovine serum free MEM containing 5 g/ml Rh123 for 1 h, trypsinized, washed with PBS and resuspended in 1 ml of MEM containing 5 g/ml PI. The intensity of fluorescence from Rh123 was analyzed by flow cytometry.

Statistical Analysis

All data are presented as mean $\pm$ SE and are the results of three experiments. ANOVA Single factor analysis was employed to calculate the statistical significance between non-treated (DMSO) C34 or E47 cells, GA treated C34 or E47 cells, SB203580 treated C34 or E47 cells or GA plus SB203580 treated C34 or E47 cells respectively. $p < 0.05$ was considered to be statistically significant.

RESULTS

SB203580 prevents cytotoxicity of GA in E47 cells

To assess the effectiveness of SB203580 in preventing the cytotoxicity of GA in HepG2 cells, different methods to assay cell viability were used. Incubation of HepG2 cells with 0.5 M GA for 36h resulted in negligible toxicity in C34 cells (6%) but a 55% decrease in viability in E47 cells as shown by the results of a MTT assay was found (Fig. 1A) confirming previous data [22]. SB203580 alone was not toxic to either C34 or E47 cells (85-90% viability), however, it lowered the toxicity of GA in the E47 cells, restoring viability to the control DMSO levels (89% viability). Treatment with GA increased trypan blue uptake 1.5 fold and 7 fold in C34 and E47 cells, respectively (Fig. 1B). SB203580 treatment completely prevented the increase in trypan blue uptake produced by GA in the C34 or E47 cells.

A small increase in leakage of LDH was observed in GA treated C34 cells, whereas LDH leakage increased 4.5-fold in E47 cells treated with GA (Fig. 1C). SB203580 alone did not cause any increase in LDH leakage in C34 or E47 cells, however, SB203580 caused a significant decrease in the GA mediated LDH leakage in E47 cells when compared to GA treated cells.

The possibility that the minimal but significant toxicity observed in C34 cells with GA could be due to the presence of CYP isoforms other than CYP2E1 in these cells was negated by the use of metyrapone or 1-aminobenzotriazole (0.1mM-0.5mM), non-specific CYP inhibitors which did not prevent GA toxicity in C34 cells (data not shown). The residual GA toxicity observed in C34 cells may reflect other nonspecific CYP-independent actions of GA which were not evaluated in this study.

Visualizing the cells under the light microscope revealed that GA treatment for 36h caused significant changes in the morphology of the E47 cells which included detachment, shrinkage and dispersed distribution of the cells, accompanied by the lack of formation of a monolayer (data not shown). Much fewer E47 cells were observed after the GA treatment. These changes were not very conspicuous in C34 cells treated with GA for the same duration. Treatment with SB203580 alone did not cause any significant morphological changes in E47 cells. When SB203580 was added to the GA-treated E47 cells, minimal changes in morphology were observed and the morphology of the cells closely resembled the morphology of untreated E47 cells (data not shown).

Characterization of the protective effect of SB203580 in GA treated E47 cells

Only 25% E47 cells were viable after GA treatment, and viability was increased to 40-50% at concentrations of 1 or 2.5 M SB203580. Higher concentrations of SB203580 i.e. 5 to 12 M conferred 55-60% protection against GA toxicity in E47 cells (Fig. 2A). GA treated C34 cells remained viable (77% of control) and the viability ranged from 78-87% at concentrations of 1 to 12 M of SB203580.

As shown in Fig. 2B, when added 1h before GA treatment, SB203580 exhibited a time dependent protective effect against GA mediated toxicity in the E47 cells. GA did not affect the viability of E47 cells during the initial 9h of treatment. At 24-30h of GA treatment, only 60-65% E47 cells survived (as compared to 78-80% viable C34 cells - data not shown). After treatment with GA for 36h the viability of E47 cells was lowered to 38% (Fig. 2B), while that of C34 cells was lowered to 76% (data not shown). At all time points of treatment from 0h to 36h, SB203580 was not toxic to either C34 or E47 cells. SB203580 restored the viability of E47 cells treated with GA; the 60-65% decrease in viable E47 cells treated with GA for 24-30h was blunted with SB203580 treatment to 80-85% viability. At 36h, the viable number of GA treated E47 cells increased from 38% to 85% with SB203580 treatment (Fig. 2B).

In the above experiments, the protective effect of SB203580 on GA cytotoxicity in HepG2 cells was evaluated with the addition of SB203580 1h before GA treatment. We examined the effects of addition of SB203580 after treatment of the E47 cells with GA. SB203580 was added at 5 min, 10 min, 15 min, 30 min, 1h, 2h, 4h, 6h, 9h, 24h, 30h and 36h after GA treatment and cells incubated for up to 36h, followed by MTT assay. SB203580, under these conditions did not increase the viability of E47 cells, even when added 5 min after GA (data not shown). Thus, SB203580 cannot prevent toxicity after E47 cells have already been treated with GA, suggesting that even a short treatment with GA, e.g. 5 min, is sufficient to initiate the events leading up to cell toxicity.

Effects of LY294002, PD98059 or SP600125 on cytotoxicity of GA in E47 cells

The effect of LY294002, a selective inhibitor of PI3 kinase, PD98059, an inhibitor of ERK MAPK and SP600125, an inhibitor of JNK activation in preventing GA mediated toxicity in E47 cells was also examined. The 65% decrease in viability in E47 cells due to GA treatment was not affected by LY294002 (Fig. 2C). Similarly, PD98059 did not significantly increase the viability of GA treated E47 cells (Fig. 2C). Addition of SP600125 also did not affect the viability of the cells significantly (Fig. 2C). Treatment with 5 M LY294002 or 10 M PD98059 or 2.5 M SP600125 alone for 36h was not toxic to the E47 cells (88-98% viability).

SB203580 decreases the GA mediated apoptotic mode of cell death in E47 cells

Treatment of C34 cells with GA caused an insignificant increase in early apoptotic cells (1.3 fold) (lower right Annexin V (+) PI (-) quadrant) when compared to the untreated cells or C34 cells treated with SB203580 alone as detected by flow cytometry (Fig. 3). In GA treated E47 cells, there was a 2 to 3 fold increase in percentage of apoptotic cells in the Annexin V (+) PI (-) (lower right quadrant) field, when compared to the untreated E47 cells or E47 cells treated with SB203580 (Fig. 3). Non significant increases were observed in the percent necrotic cells (upper left or right quadrants reflecting PI (+) cells) or late apoptotic / necrotic cells (upper right Annexin V (+) PI (+) quadrant). Thus, the GA- mediated toxicity in the E47 cells is apoptotic in nature. SB203580 was effective in decreasing this percentage of apoptotic cells when compared to GA treated E47 cells (Fig. 3).

SB203580 decreases the GA mediated DNA fragmentation in E47 cells

The percentage of apoptotic HepG2 cells due to GA treatment was measured by analyzing the DNA distribution of C34 and E47 cells by flow cytometry. There was a 3-fold and a 6-fold increase in the subG₀/G₁ fraction (M1 zone, hypodiploid area) in the C34 and E47 cells respectively after GA treatment (Fig. 4). SB203580 alone caused insignificant increases in the percentage of HepG2 cells in the M1 phase. Addition of SB203580 to GA treated E47 cells resulted in large (70%) reduction in the percentage of E47 cells in the M1 phase (Fig. 4).

GA mediated activation of p38 MAPK is an early and sustained response in E47 cells

The decrease in GA mediated toxicity in the E47 cells in the presence of SB203580, suggests the involvement of the p38 MAPK pathway in GA mediated toxicity. p38 MAPK is activated primarily through its phosphorylation. Treatment of E47 cells with GA resulted in an increase in the phosphorylation of p38; however the extent of activation as reflected by the pp38/p38 MAPK ratio was less in C34 cells (Figs. 5A&B). GA treated E47 cells had 3-4 fold higher pp38/p38 ratios than the non-treated E47 cells during a 15min-4h incubation period; this increase in pp38 decreased to the basal level at 36h. The pp38 to p38 ratio increased in GA treated C34 cells about 1.5 fold during the first 15 min of treatment but then rapidly declined to control levels or even lower at 30 min and 1 to 4h of incubation and decreased rapidly at 36h (Figs. 5A & B). Thus, the increase in p38 MAPK activation by GA was greater in the E47 cells, and was also sustained for a more prolonged period than that found with the C34 cells.

Treatment of HepG2 cells with GA for 15 minutes resulted in almost 2 fold increase in the ratio of phosphorylated p38 to p38 in E47 cells (Fig. 6). SB203580 alone caused a small decrease in the pp38 levels in E47 cells during the 15 minute incubation period when compared to the untreated HepG2 cells. Addition of SB203580 to the GA treated E47 cells caused a decline in the pp38/p38 ratio back to basal levels, i.e., the GA-mediated increase in p38 MAPK activation was abrogated by SB203580 in the E47 cells.

Effect of SB203580 on GA mediated ROS production, lipid peroxidation and GSH levels in HepG2 cells

We previously showed that the enhanced toxicity of GA in E47 cells compared to C34 cells was associated with increased oxidant stress [22]. The possible role of p38MAPK in this elevated generation of ROS, and lipid peroxidation and decline in GSH levels in E47 cells treated with GA was studied. ROS levels were higher in E47 cells than C34 cells (Fig. 7A). Treatment of C34 cells with GA increased ROS levels 1.3 fold, whereas, ROS levels were elevated 3-fold in GA treated E47 cells (Fig. 7A). SB203580 alone did not affect ROS levels in C34 or E47 cells. SB203580 lowered ROS almost back to the basal levels of untreated E47 cells, i.e., most of the increase in DCF fluorescence produced by GA was prevented by SB203580.

MDA formation was higher in DMSO treated control E47 cells compared to C34 cells treated with DMSO (Fig. 7B). Lipid peroxidation was only increased slightly in GA treated C34 cells, however, MDA formation was increased more than 2-fold by GA in the E47 cells (Fig. 7B). The addition of SB203580 alone had no effect on basal lipid peroxidation values, however, SB203580 addition to GA treated E47 cells caused a decrease in the GA-stimulated lipid peroxidation (Fig. 7B).

GA slightly decreased the GSH levels in C34 cells when compared to the DMSO treated C34 cells and SB203580 alone or in the presence of GA had little effect on GSH levels (Fig. 7C). E47 cells had a 2.5 fold higher GSH content than the C34 cells as previously shown [29]. This increase in GSH was suggested to reflect a metabolic adaptation by the E47 cells to the CYP2E1-oxidant stress [29]. Treatment with GA caused a striking decrease in the GSH levels

in the E47 cells. In the presence of SB203580, this decrease in GSH content in GA-treated E47 cells was partially, but not completely prevented (Fig. 7C).

SB203580 prevents the decline in mitochondrial membrane potential in GA treated E47 cells

C34 and E47 cells showed a decline in mitochondrial membrane potential after GA treatment as evidenced by the increased percentage of cells with low rhodamine fluorescence (M1 cells) (Fig. 8 -2 fold increase in M1 C34 cells and 4-fold increase in M1 E47 cells). Exposure of the HepG2 cells to SB203580 alone for 36h did not cause a decrease in mitochondrial membrane potential. SB203580 was effective in preventing the decrease in mitochondrial membrane potential in the GA- treated E47 cells as the number of cells in the M1 phase was decreased 2 fold (Fig. 8).

Effect of SB203580 on CYP2E1 protein expression and activity and Hsp90 expression in HepG2 cells

As expected, E47 cells expressed high CYP2E1 protein content while the C34 cells did not express the protein (Fig. 9A). SB203580 slightly but not significantly decreased the expression of CYP2E1 when compared to the untreated E47 cells and had no effect in C34 cells (Fig. 9A). Similarly, CYP2E1 catalytic activity as reflected by the oxidation of 7-MFC was much higher in the E47 cells compared to the C34 cells and SB203580 had no effects on this oxidation (numbers above the immunoblots, Fig. 9A).

Western blot and densitometric analysis of Hsp90 with and without GA or SB203580 treatment in HepG2 cells is shown in Fig. 9B. The HepG2 cells expressed high levels of Hsp90 and this expression was 2 fold higher in E47 cells when compared to C34 cells. GA or SB203580 alone did not significantly affect the levels of Hsp90 in C34 or E47 cells. Hsp90 levels were not affected in GA plus SB203580 treated C34 cells but the combination of GA and SB203580 caused a small decrease (20%) of the Hsp90 level in E47 cells.

DISCUSSION

Hsp90 is a ubiquitous cellular protein and its functions as a molecular chaperone are vital for cell survival [4-8]. Hsp90 mediates the proper folding and prevention of aggregation of numerous proteins in the cell [6-8]. It has a wide substrate specificity ranging from steroid hormone receptors, transcription factors, kinases and polymerases [3,30]. Hsp90 is also essential for the activation of many regulatory and signaling proteins [3,6-8,13-15]. The conversion of extracellular stimuli into appropriate intracellular responses is mediated through the coordinated actions of proteins involved in the signal transduction pathway. Proteins involved in major signal transduction pathways also include the mitogen-activated protein kinases, MAPK [12]. Hsp90 has been shown to stabilize cell signaling proteins and the binding of HSP90 to these proteins helps in the folding, conformational maturation, translocation and formation of immobilized signal transduction complexes [8,16,30-32]. p38 MAPK appears to be stimulated primarily through environmental stress and cytokines and is inhibited by the anti-inflammatory drug SB203580 which has been used extensively to delineate this signaling pathway [12,33-35].

GA, an inhibitor of Hsp90 is a commonly used anti-tumor drug which is effective due to the essential role of Hsp90 in the proliferation and survival of cancer cells [9-11]. The chaperone functions of Hsp90 depend on its ATP binding and hydrolyzing ability and GA binds at its ATP-binding site, thus acting as a competitive inhibitor and affecting Hsp90 binding affinity to substrates and co-chaperones [9-11]. Several studies have shown that GA disrupts the complex formation of Hsp90 with signal transduction proteins and thus interferes with pathways involving these proteins [36-38]. CYP2E1 is a major source of oxidative stress in

cells and previously, we have shown that GA potentiates CYP2E1 toxicity in HepG2 cells [22]. GA also disrupts the binding of Hsp90 to kinases involved in signal transduction pathways [30]. However, the possible role of MAP kinases involved in signal transduction pathways in GA mediated regulation of CYP2E1 toxicity in HepG2 cells has not been studied.

In the present study GA was toxic to E47 cells and SB203580 increased the viability and prevented the aberrations in morphology in E47 cells due to GA treatment. These results suggest a role of the p38MAPK pathway in GA mediated toxicity in E47 cells. The protective effects of SB203580 were observed only when the E47 cells were pretreated with the p38 MAPK inhibitor before subsequent addition of GA. When SB203580 was added after GA addition, the decline in viability of E47 cells was not prevented. Even addition of SB203580 as early as 5 min after GA failed to protect suggesting that an early activation of p38 MAPK is a prerequisite for GA toxicity in the HepG2 cells and the inhibition of p38 MAPK does not protect the cells from CYP2E1 mediated toxicity once GA initiated its effects.

Since the inhibition of p38MAPK resulted in prevention of the toxicity of GA in the E47 cells, it was important to examine whether inhibition of Hsp90 with GA leads to the activation of p38MAPK, as measured by the increase in the phosphorylated to the non-phosphorylated ratio. Indeed, the pp38 MAPK levels were increased from 15min-4h after GA addition and decreased to the basal levels at 36h in the E47 cells, indicating that the activation of p38 MAPK is an early event which eventually leads to cell toxicity during further incubation. The GA treated E47 cells showed elevated and especially more sustained activation of pp38 MAPK when compared to the C34 cells, suggesting that potentiation of CYP2E1 toxicity in the E47 cells is due, in part to a loss of the protective functions of Hsp90 by GA treatment coupled to p38 MAPK activation.

Hsp90 has been shown to play an anti-apoptotic role [39-42] and our previous study has shown that inhibition of Hsp90 with GA increases apoptosis in E47 cells [22]. In the present study, inhibition of p38 MAPK with SB203580 decreased the percentage of apoptotic E47 cells significantly, as assessed by Annexin V staining or DNA analysis, consistent with other studies where the p38 MAPK inhibitor SB203580 reversed apoptosis [43,44].

We also investigated the involvement of other kinase pathways in mediating the toxic effects of GA in E47 cells using LY294002, a PI3 kinase inhibitor, PD98059, an ERK MAPK inhibitor and SP600125, a JNK inhibitor. These inhibitors were found to be ineffective in preventing GA mediated toxicity in E47 cells, suggesting that PI3 kinase, ERK MAPK and JNK pathways are not involved in the GA-mediated toxicity.

We have previously shown that GA increases ROS levels and lipid peroxidation and decreases GSH content in E47 cells [22]. We hypothesized that GA inhibition of Hsp90 function lowered cellular protection against CYP2E1 resulting in an increase in CYP2E1- generated oxidant stress by GA. Indeed, the 2-fold elevation in Hsp90 content in E47 compared to C34 cells like the increase in GSH and other antioxidants [29] reflect metabolic adaptations by the E47 cells to remove the CYP2E1 oxidant stress. Activation of p38MAPK by GA may be mediated by the increased oxidative stress produced by GA, or the activation of MAPK by GA may be important in the developing oxidative stress. The inhibition of p38 MAPK with SB203580 lowered ROS and malondialdehyde accumulation and increased the levels of GSH in GA treated E47 cells. A recent report demonstrated that SB203580 protects dopaminergic neurons against interferon-LPS injury by the suppression of nitric oxide generation [45]. In a recent study using HeLa cells, the addition of H₂O₂ did not affect activation of p38 MAPK, suggesting that p38 MAPK functions upstream of ROS [46]. The reduction in ROS and lipid peroxidation and the partial restoration of GSH in SB203580 treated E47 cells may indicate that the elevated ROS functions downstream of MAPK pathways and the GA-mediated inhibition of Hsp90 in

CYP2E1 over-expressing E47 cells leads to oxidative stress. The suppression of activated p38 MAPK with SB203580 downregulates the increased oxidant stress due to GA treatment and protects against loss of cell viability.

Another possible explanation for the lowering of the GA-induced elevated oxidant stress by SB203580 may reflect the effects of SB203580 on CYP2E1 or Hsp90 levels. However, SB203580 had no effect on the levels or activity of CYP2E1 in the E47 cells. Hsp90 levels were elevated in the E47 cells compared to the C34 cells. GA caused an insignificant decrease in Hsp90 levels in the E47 cells, and while a decrease in Hsp90 would promote an increase in oxidative stress, the extent of decrease does not appear to be sufficient to account for the elevated oxidant stress. SB203580 did not alter basal Hsp90 levels significantly in the E47 or C34 cells. The SB203580 mediated prevention of the GA-induced increase in toxicity is not at the levels of CYP2E1 or Hsp90.

Damage to the mitochondria and consequent decline in mitochondrial membrane potential plays an important role in apoptosis [47] and CYP2E1-mediated toxicity [48]. GA caused mitochondrial damage in E47 cells, and to a lesser extent in C34 cells, as assessed by loss in mitochondrial membrane potential. SB203580 partially reversed the mitochondrial damage in the E47 cells. Mitochondrial dysfunction has been shown to activate MAPK pathways [49] but activated p38 MAPK may cause mitochondrial dysfunction [34,50]. The decrease in percentage of cells with low mitochondrial membrane potential after SB203580 treatment suggests that GA toxicity in CYP2E1 over-expressing cells through a p38 MAPK pathway and perhaps increased oxidant stress may cause damage to the mitochondria. Alternatively, mitochondrial damage due to CYP2E1 plus GA toxicity may activate p38 MAPK. Further studies will be needed to evaluate whether activated p38 MAPK is upstream of the mitochondrial damage or is a result of the mitochondrial damage.

In summary, the p38 MAPK inhibitor, SB203580, prevents GA toxicity in E47 cells indicating that the potentiation of toxicity due to Hsp90 inhibition by GA in HepG2 cells over-expressing CYP2E1 is mediated through the activation of a p38 MAPK pathway. The addition of SB203580 prior to GA treatment was critical for its protective effects. Short-term treatment with GA caused activation of p38 MAPK in the E47 cells. SB203580 decreased the GA-induced elevation of ROS levels and lipid peroxidation, and increased GSH levels in the E47 cells. The increase in number of GA treated cells showing an apoptotic mode of cell death and low mitochondrial membrane potential were decreased significantly with SB203580 treatment. The results suggest that the Hsp90 inhibitor GA potentiates CYP2E1 toxicity through a p38 MAPK pathway.

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Abbreviations

ANOVA, analysis of variance; BHT, butylated hydroxytoluene; C34 cells, HepG2 cell line established after transfection with pCI-neo; CYP2E1, cytochrome P4502E1; DCF-DA, 2',7'-dichlorofluorescein diacetate; DMSO, dimethyl sulfoxide; E47 cells, HepG2 cell line established after transfection with pCI-neo-CYP2E1; ERK, extracellular signal-regulated kinase; FITC, fluorescein isothiocyanate; GA, geldanamycin; GSH, reduced glutathione; Hsp, Heat shock protein; JNK, c-Jun NH₂-terminal kinase; LDH, lactate dehydrogenase; LY294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; MAPK, mitogen-activated protein kinase; MEM, minimal essential medium; MFC, 7-methoxy-4-trifluorocoumarin; MTT, 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered

saline; PD98059, 2'-amino-3'-methoxyflavone; PI, propidium iodide; PI3K, phosphatidylinositol 3-kinase; Rh123, rhodamine 123; ROS, reactive oxygen species; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole.

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Fig 1.

Effect of SB203580 on the viability of C34 and E47 cells in the absence and presence of GA. C34 or E47 cells were treated with 0.1% DMSO or 0.5 M GA or 10M SB203580 or 10M SB203580 plus 0.5 M GA for 36 h and cell viability was assessed by (A) the MTT assay, (B) trypan blue test and (C) LDH leakage assay. Results are expressed as mean $\pm$ SE of three independent experiments. *, $p < 0.05$ for C34+GA or E47+GA compared to C34+DMSO or E47+DMSO; #, $p < 0.05$ for C34+GA or E47+GA compared to C34+SB or E47+SB; Φ, $p < 0.05$ for E47+GA compared to C34+GA and ‡, $p < 0.05$ for C34+SB+GA or E47+SB+GA compared to C34+GA or E47+GA.

Fig 2.

(A). Effects of different concentrations of SB203580 on the viability of GA treated C34 and E47 cells. C34 or E47 cells were pretreated with 0.1% DMSO or 0.5 M GA or 1 to 12 M SB203580 plus 0.5 M GA for 36h and cell viability was assessed by the MTT assay. Results are expressed as mean $\pm$ SE of three independent experiments. *, $p < 0.05$ for C34+GA or E47+GA compared to C34+DMSO or E47+DMSO and ‡, $p < 0.05$ E47+SB+GA compared to E47+GA. (B) Time-dependent effects of SB203580 on the viability of GA treated C34 and E47 cells. SB203580 was added 1h before GA treatment of HepG2 cells. C34 or E47 cells were pretreated with 0.1% DMSO or 0.5 M GA or 10 M SB203580 plus 0.5 M GA for 30min, 1, 2, 4, 6, 9, 24, 30 or 36h. Cell viability was assessed by the MTT assay. Results are expressed as mean $\pm$ SE of three independent experiments. (C) Effect of LY294002, a PI3 kinase inhibitor, PD98059, an ERK MAPK inhibitor and SP600125, an inhibitor of JNK on the viability of GA treated E47 cells. E47 cells were pretreated with 0.1% DMSO or 0.5 M GA or 5 M LY294002 or 5 M LY294002 plus 0.5 M GA or 10 M PD98059 or 10 M PD98059 plus 0.5 M GA or 2.5 M SP600125 or 2.5 M SP600125 plus 0.5 M GA for 36h. Cell viability was assessed by the MTT assay. Results are expressed as mean $\pm$ SE of three independent experiments. *, $p < 0.05$ for E47+GA compared to E47+DMSO and #, $p < 0.05$ for E47+GA compared to E47+LY/PD/SP.

Fig 3.

Effect of SB203580 on apoptosis produced by GA in C34 and E47 cells. After treatment with 0.5 M GA or 10 M SB203580 or 10 M SB203580 plus 0.5 M GA for 36h, C34 and E47 cells were stained with PI and Annexin V conjugated with FITC, and then analyzed by flow cytometry. Upper quadrants reflect PI positive cells; lower right quadrant reflects AnnexinV positive cells.

Fig 4.

Effect of SB203580 on GA-induced DNA fragmentation in C34 and E47 cells. HepG2 cells were pretreated with 0.5 M GA or 10 M SB203580 or 10 M SB203580 plus 0.5 M GA for 36h and the cells were harvested by trypsinization, fixed with ethanol, and stained with PI. The cells were then analysed using flow cytometry and their representative histograms are shown. The percentage of cells in the sub- G_0/G_1 fraction (M1 zone, hypodiploid area) is shown.

Fig 5.

(A) Effect of GA on p38 and pp38 MAPK levels in C34 and E47 cells. C34 or E47 cells were treated with 0.1% DMSO or 0.5 M GA for 15min, 30 min, 1, 2, 4, 36 h and p38 and pp38 MAPK proteins were detected by Western blotting. One representative Western blot out of three blots showing pp38 and p38 MAPK protein bands in HepG2 cells. (B) Densitometric values (du) for pp38 to p38 ratios in HepG2 cells. Results are from three blots. *, $p < 0.05$ for C34+GA or E47+GA compared to C34+DMSO or E47+DMSO. Two separate gels are shown: one reflects the results of panels 1-8 and the second gel, panels 9-16. All quantification is the ratio of pp38/p38.

Fig 6.

(A) Effect of SB203580 on p38 and pp38 MAPK levels in C34 and E47 cells. C34 or E47 cells were treated with 0.1% DMSO or 0.5 M GA for 15min and p38 and pp38 MAPK proteins were detected by Western blotting. One representative Western blot out of three blots showing p38 MAPK protein bands in C34+DMSO (lane 1), C34+GA (lane 2), C34+SB203580 (lane 3), C34+SB203580+GA (lane 4), E47+DMSO (lane 5), E47+GA (lane 6), E47+SB203580 (lane 7) and E47+SB203580+GA (lane 8); (B) Densitometric values (du) for pp38 to p38 ratios in HepG2 cells. *, $p < 0.05$ for C34+GA or E47+GA compared to C34+DMSO or E47+DMSO; †, $p < 0.05$ for E47+SB compared to E47+DMSO; #, $p < 0.05$ for C34+GA or E47+GA compared to C34+SB or E47+SB; Φ, $p < 0.05$ for E47+GA compared to C34+GA and ‡, $p < 0.05$ for C34+SB+GA or E47+SB+GA compared to C34+GA or E47+GA.

Fig 7.

Effect of SB203580 on ROS levels, lipid peroxidation and GSH in HepG2 cells. C34 or E47 cells were pretreated with 0.1% DMSO or 0.5 M GA or 10 M SB203580 or 10 M SB203580 plus 0.5 M GA for 36h. (A) The levels of intracellular ROS were measured by fluorescence spectrophotometry using 2', 7'-DCF-DA as the probe. (B) Lipid peroxidation was evaluated by measuring the amounts of thiobarbituric acid-reactive components formed as determined by the malondialdehyde equivalents. (C) The total GSH content of samples was assayed by measuring the rate of 2-nitro-5-thiobenzoic acid production. Results are expressed as mean $\pm$ SE of three independent experiments. *, $p < 0.05$ for C34+GA or E47+GA compared to C34+DMSO or E47+DMSO; †, $p < 0.05$ for E47+DMSO compared to C34+DMSO; #, $p < 0.05$ for C34+GA or E47+GA compared to C34+SB or E47+SB; Φ, $p < 0.05$ for E47+GA compared to C34+GA and ‡, $p < 0.05$ for E47+SB+GA compared to E47+GA.

Fig 8.

Effect of SB203580 on mitochondrial membrane potential in C34 and E47 cells. C34 or E47 cells were pretreated with 0.1% DMSO or 0.5 M GA or 10 M SB203580 or 10 M SB203580 plus 0.5 M GA for 36h. Representative histograms of the measurement of mitochondrial membrane potential as monitored by flow cytometry using Rh123 are shown. RHO stands for Rh123 fluorescence. The percentage of cells in the M1 fraction (low rhodamine 123 fluorescence) are represented by the numerical values.

Fig 9.

(A) Effect of SB203580 on CYP2E1 levels and activity in C34 and E47 cells. C34 or E47 cells were treated with 0.1% DMSO or 10M SB203580 for 36 h and the CYP2E1 protein level was detected by Western blotting. Left: Representative Western blots showing CYP2E1 protein bands in E47+DMSO (lane 1), E47+SB203580 (lane 2), C34+DMSO (lane 3) and C34+SB203580 (lane 4); right: Densitometric values (du) for CYP2E1 in HepG2 cells. CYP2E1 catalytic activity was measured with determination of the fluorescence generated through the hydrolysis of 7-methoxy-4-trifluorocoumarin 409/530 nm and results are expressed above the immunoblots as arbitrary units of fluorescence. (B) Effect of SB203580 on Hsp90 levels in C34 and E47 cells. C34 or E47 cells were treated with 0.1% DMSO or 0.5 M GA or 10M SB203580 or 10M SB203580 plus 0.5 M GA for 36 h and the Hsp90 protein level in C34 and E47 cells was detected by Western blotting. Left: One representative Western blot out of three blots showing Hsp90 protein bands in C34+DMSO (lane 1), C34+GA (lane 2), C34+SB203580 (lane 3), C34+SB203580+GA (lane 4), E47+DMSO (lane 5), E47+GA (lane 6), E47+SB203580 (lane 7) and E47+SB203580+GA (lane 8); right: Densitometric values (du) for Hsp90 in HepG2 cells. *, $p < 0.05$ for E47+SB+GA compared to E47+DMSO or E47++SB and †, $p < 0.05$ for E47+DMSO compared to C34+DMSO.