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Role of oxidative stress in Geldanamycin-induced cytotoxicity and disruption of Hsp90 signaling complex

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Abstract

Heat shock protein 90 (Hsp90) is a chaperone protein regulating PC-12 cell survival by binding and stabilizing Akt, Raf-1, and Cdc37. Hsp90 inhibitor Geldanamycin (GA) cytotoxicity has been attributed to disruption of Hsp90 binding, and the contribution of oxidative stress generated by its quinone group has not been studied in this context.

Reactive oxygen species (ROS) and cell survival were assessed in PC-12 cells exposed to GA or Menadione (MEN), and Akt, Raf-1, and Cdc37 expression and binding to Hsp90 were determined. GA disrupted Hsp90 binding and increased ROS production starting at 1h, and cell death occurred at 6h, inhibited by N-acetyl cysteine (NAC) without preventing dissociation of proteins. At 24h, NAC prevented cytotoxicity and Hsp90 complex disruption. However MnTBAP antioxidant treatment failed to inhibit GA cytotoxicity, suggesting that NAC acts by restoring glutathione. In contrast, 24h MEN induced cytotoxicity without disrupting Hsp90 binding. GA and MEN decreased Hsp90-binding proteins expression, and proteasomal inhibition prevented MEN, but not GA-induced degradation. In conclusion, while MEN cytotoxicity is mediated by ROS and proteasomal degradation, GA-induced cytotoxicity requires ROS but induces HSP90 complex dissociation and proteasome-independent protein degradation. These differences between MEN and GA-induced cytotoxicity, may allow more specific targeting of cancer cells.

Keywords

Heat shock protein 90; Geldanamycin; Reactive oxygen species; Protein degradation

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Introduction

Heat shock proteins (Hsps) are conserved chaperones that stabilize proteins, refold misfolded proteins, or target irreversibly damaged proteins to degradation [1–5]. In addition, Hsp function is also critical to subcellular targeting of newly synthesized proteins [6]. Hsps regulate proliferation, cell cycle, and pro- and anti-apoptotic pathways and are implicated in multiple pathologies [1–5]. Hsp90 is a highly abundant protein that regulates a broad spectrum of biological processes by interacting with various kinases, co-chaperones, transcription factors, and other binding proteins to properly fold and stabilize them [7,8]. Therefore, Hsp90 protein complexes are implicated in multiple physiological processes and are critical to cell survival.

The protein interactions of Hsp90 are regulated by ATP binding to the N-terminal domain of Hsp90, altering the conformation of Hsp90 dimers and allowing proteins to bind tightly to Hsp90. The ATP-dependent chaperone cycle of Hsp90 is critical to the maturation and stabilization of its binding proteins [9,10]. Geldanamycin (GA), a benzoquinone ansamycin compound, inhibits the chaperone function of Hsp90 by competing with ATP for binding to the ATP-binding pocket of Hsp90, and prevents Hsp90 protein binding [11,12].

Hsp90 binds to Akt, Raf-1, an upstream activator of the Raf/MEK/ERK kinase pathway, and to Cdc37, a kinase co-chaperone, regulating Akt and Raf-1 survival pathways [13–17]. Although Hsp90 is abundantly expressed in all eukaryotic cells, its expression and that of its binding proteins is particularly high in cancer cells. Therefore, cancer cells may rely more heavily on Hsp90 complexes for survival, and may be more sensitive to Hsp90 inhibitors, simultaneously targeting many Hsp90-dependent oncogenic pathways [18]. Indeed, vaccines and other novel therapies targeting cancer cells Hsp90 complexes were developed to increase the immune response to tumors [19,20] and several GA derivatives entered clinical trials as adjuvants to conventional chemotherapy [21–24]. However, GA and its derivatives are toxic to normal cells, limiting the doses and durations of treatments that can be administered to patients.

Although GA cytotoxicity has been attributed to its disruption of Hsp90 complexes, this compound also carries a benzoquinone moiety that generates ROS [25,26]. Additionally, GA has been found to react non-enzymatically with glutathione (GSH) at physiological pH and concentrations, forming stable GSH adducts and depleting cellular GSH [27]. Oxidative stress may lead to cellular stress and demise via protein oxidation and degradation. Moreover, GA reaction with thiol groups of critical proteins may enhance its cytotoxicity. However, the potential role of the GA benzoquinone group in GA cytotoxicity and in disruption of Hsp90 binding has not been examined. To study the role of oxidative stress in GA-induced cell death and in the disruption of the Hsp90 complex, and its effect on Hsp90-binding proteins stability, PC-12 cells were treated with GA in combination with antioxidants. To this effect, we used both the glutathione precursor N-acetyl cysteine (NAC), as GA also causes GSH depletion, and a superoxide dismutase mimetic and scavenger of peroxynitrite, 4[Mn (III)tetrakis(4-Benzoic acid) porphyrin Chloride] (MnTBAP) [28], as GA has been shown to generate superoxide [25–27]. Additionally, we examined the effect of proteasomal and lysosomal degradation inhibitors on GA-induced dissociation of Hsp90 complex, as these degradation pathways are implicated in the removal of oxidized proteins. The classical quinone menadione (MEN) was used as a positive control as similarly to GA in MEN harbors one reactive quinone group that produces ROS in cells, decreases NADPH, and non-enzymatically forms covalent conjugates with the cysteine residues of glutathione. MEN has recently been used as an enhancer of anticancer agents [27,29–31]. Our findings reveal that while oxidative stress alone is insufficient to disrupt Hsp90 binding to Akt, Raf-1, and Cdc37, prolonged exposure alters their expression. In contrast to MEN, GA first disrupts protein binding to Hsp90 and induces oxidation of the released proteins, leading to their degradation and to PC-12 cell death. NAC

but not MnTBAP rescues cells from GA-induced cytotoxicity however NAC does not prevent early protein dissociation implicating GSH depletion in GA cytotoxicity suggesting that protein dissociation is independent from oxidative stress. This early GA-induced dissociation of the Hsp90 complex may enhance the sensitivity to GA of Hsp90-enriched cancer cells. While MEN-induced ROS and protein degradation responses are dependent on activation of the proteasomal pathway, GA-induced degradation appears to be mediated by an alternative degradation pathway. These findings suggest that disrupting Hsp90 binding results in the targeting of oxidized proteins to degradation, inducing cell death, even when the proteasomal pathway is inhibited. Therefore, GA may be used as an early therapeutic option, allowing for targeting of cancer cells, and followed by the use of a non-toxic adjuvant drug to limit protein degradation and cell death in non-cancerous cells.

Experimental procedures

Materials

All reagents were from Sigma (St Louis, MO) unless otherwise specified. Cell culture media and additives were from Invitrogen (Carlsbad, CA). Trypan Blue (0.4%; J. T. Baker Chemical Company, Phillipsburg, NJ) and 3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Calbiochem, San Diego, CA) were dissolved in phosphate buffered saline (PBS). Geldanamycin (GA; LC Laboratories, Inc., Woburn, MA), Menadione (MEN), MG-132 (Calbiochem), Bafilomycin A₁ (BAF; Calbiochem), and 2',7'-dichlorofluorescein (DCF; Molecular Probes, Eugene, OR) and Dihydroethidium (DHE, Calbiochem), were dissolved in sterile DMSO. N-acetyl cysteine (NAC) and 4[Mn (III)tetrakis(4-Benzoic acid)porphyrin Chloride] (MnTBAP) were dissolved in supplemented RPMI1640 culture media.

Cell culture

Rat pheochromocytoma PC-12 cells, grown at 37°C in 5% CO₂ incubators, were cultured on rat tail collagen-coated plates in RPMI1640 medium supplemented with 10% horse serum, 5% fetal bovine serum, and 1% antibiotics/antimycotics. Cell confluence was estimated by light microscopy and by MTT assay.

Cell exposures

Cells were grown to 70–80% confluence for NAC, BAF, MEN, Mn-TBAP and GA treatments, and to 40–50% for MG-132 pre-treatments, that required a longer incubation. To determine the time course and dose of GA cytotoxicity, cell viability was assessed after 1h, 3h, 6h, 9h, 12h, or 24h with 10 or 20 µM GA or DMSO vehicle. To assess the role of oxidative stress in GA cytotoxicity, cells were pretreated with 1 mM NAC or vehicle for 1h, followed by 1h, 6h, or 24h treatments with 20 µM MEN, 20 µM GA, or vehicle. In additional experiments cells were pretreated with 40µM MnTBAP prior to their exposure to 20 µM GA. For proteasome inhibition studies, cells were pre-treated 21h with 150 nM MG-132 or DMSO, followed by 1h, 9h, or 27h treatments with 20 µM GA, 20 µM MEN, or DMSO. For lysosomal protein degradation studies, cells were pre-treated for 30 min with up to 750 nM BAF and treated for 6h with 20 µM GA or DMSO. Following exposures, cell viability, ROS production, protein expression, and Hsp90 binding were assessed as described below.-

Cell viability

Viability was measured by MTT or by Trypan blue exclusion assays. MTT assay was performed by adding formazan dye MTT (3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) 1h prior to cell collection. Cell pellets were washed, reduced formazan products were solubilized in DMSO, and optical density was measured at 450 nm. Trypan blue exclusion assay was performed by counting Trypan blue stained cells on a

hemacytometer using a light microscope connected to a digital camera and computer with Motic imaging software.

Reactive oxygen species (ROS) production

Formation of intracellular oxidant species was detected by fluorescence, using the cell permeable non-fluorescent 2'-7' dichlorodihydrofluorescein diacetate (DCFH₂-DA). DCFH₂-DA is de-esterified within cells by endogenous esterases and trapped. 2'-7' Dichlorofluorescein is oxidized by intracellular ROS to fluorescent 2'-7' dichlorofluorescein. However DCF has been reported to leak and react with a host of peroxidases and ROS species, therefore DCF may serve as a general indicator of oxidative status. Thus, we have also used dihydroethidium (DHE) as a more specific indicator of superoxide formation after 1h GA treatment. DHE displays cytosolic blue fluorescence, turning to bright red fluorescence following its oxidation and intercalation to DNA preventing any leakage after its oxidation. Cells were loaded for 1h with 5 μ M DCFH₂-DA, or with 10 μ M DHE, washed and resuspended in PBS, and transferred to 96-well plates (Greiner Bio-One, Monroe, NC). Fluorescence was measured at 485 nm excitation and 535 nm emission using a TECAN SPECTRAFLUOR Plus fluorescent microplate reader.

Antibodies

Akt, phospho-Ser 473-Akt, ERK1, Cdc37, and Raf-1 primary antibodies used for immunoblotting were purchased from Santa Cruz (Santa Cruz, CA), anti-phospho-Ser473 Akt and anti-phospho-ERK1/2 were from Cell Signaling Technology (Danvers, MA), anti-Hsp90 was from Assay Designs (Ann Arbor, MI), and anti-ubiquitin and anti- β -actin were from Sigma. Secondary antibodies were anti-rat IgG HRP (horseradish peroxidase; Assay Designs, Ann Arbor, MI), anti-rabbit HRP (Cell Signaling Technology, Danvers, MA), and ExactaCruz anti-mouse and anti-rabbit HRP-conjugates were from Santa Cruz. For immunoprecipitations, Hsp90 and Cdc37 antibodies were from Santa Cruz and anti-Akt was from Cell Signaling Technology.

Immunoblotting

Cell lysates (NP-40 soluble lysates fractions) were prepared by resuspending cells in lysis buffer (0.4% NP-40, 1% glycerol, 50 mM tris at pH 7.5, 1 mM Na orthovanadate, 150 mM NaCl, 10 mM EDTA, 100 mM NaF, 20 mg/mL leupeptin, 10 mg/mL aprotinin, 0.5 mM PMSF). Protein concentrations were measured using the BioRad DC Protein Assay Kit (BioRad, Temecula, CA). Protein samples were separated on 4–12% tris-glycine gels (Invitrogen, Carlsbad, CA) and transferred onto nitrocellulose membranes for immunodetection. Proteins were visualized with ECL-Plus (General Electric, Niskayuna, NY). Equal loading was verified by reprobing the membranes with β -actin antibody. Equal transfer of proteins onto nitrocellulose membranes was verified by Ponceau-S staining. Immunoblots of lysate fractions and of immunoprecipitates were repeated at least three times.

Immunoprecipitation

Lysates were incubated with Protein A-conjugated Agarose beads (Roche Applied Science, Indianapolis, IN) and either primary immune antibody or non-immune isotype control antibody in lysis buffer with 0.4% NP-40. Immunoprecipitates were washed in lysis buffer without NP-40 and resuspended in Laemmli sample buffer for immunoblotting.

Statistical analysis

Immunoblotting and immunoprecipitation experiments shown are representative of 3–5 experiments. Measurements of cell viability and reactive oxygen species production were repeated five or more times. Statistical analysis was performed using the computer program,

Statistical Package for the Social Sciences, Version 14.0 (SPSS). Significant differences in cell viability and ROS production data were determined by one-way or two-way Analysis of variance (ANOVA), followed by Tukey HSD post-hoc t-tests.

Results

Effect of GA on PC-12 cell survival

To determine the effect of Hsp90 inhibition on PC-12 cell survival, cells were exposed to 10 and 20 μ M GA for up to 24h and cell survival was assessed by MTT assay. No significant cytotoxicity occurred at 1h and 3h treatment, and GA induced cell death by 6h. While both 10 and 20 μ M GA induced cell death, cell toxicity induced by 20 μ M GA was significantly higher when compared to the lower dose. For all further experiments, the 20 μ M dose was selected. These data suggest that GA induced cell death in a time and dose-dependent manner (Fig 1).

Effect GA on ROS production and ROS-mediated cytotoxicity

To elucidate whether the oxidative stress induced by GA quinone group underlies its cytotoxicity, ROS production was assessed, using the DCF general indicator, in GA-treated cells and compared to MEN-induced ROS at 1h, 6h, and 24h. GA significantly increased ROS at 1h (Fig 2A) while MEN did not increase ROS until 6h (Fig 2C). While GA-induced ROS returned to baseline at 24h MEN-induced ROS remained elevated (Figs 2D). In addition, ROS formation was assessed after 1h GA using DHE that more specifically detects superoxide. Confirming the data obtained with DCF, ROS significantly increased after 1 h GA using DHE probe for detection (Fig 2B). Cell survival assays showed no GA cytotoxic effect at 1h (Fig 3A), despite elevated ROS (Fig 2A, 2B). Thus, the early increase in ROS was insufficient to induce cell death at this early time point. However, significant cell death started at 6h (Fig 3B) and increased by 24h (Fig 3C). Pre-treatment with the glutathione precursor, NAC decreased GA- and MEN-induced DCF fluorescence to baseline levels and prevented cell death at all time points (Figs 2, 3B and 3C). GA has been shown to deplete glutathione. Therefore, to determine whether NAC rescue of GA-treated cells is mediated by its role as a glutathione precursor, cells were treated with MnTBAP, a superoxide dismutase mimetic that scavenges superoxide, hydrogen peroxide and peroxynitrite but does not depend on the glutathione redox cycle for its activity. MnTBAP inhibited GA-induced ROS at 1h (Fig. 2B) however was unable to prevent GA-induced cell death (Fig. 3D), suggesting that ROS scavenging is insufficient to rescue the cells from GA-induced cell death and that cell recovery with NAC may be associated to its ability to restore glutathione levels. At 24h, ROS remained at baseline levels while GA was cytotoxic. The effect of NAC on cell survival at 24h confirms that early ROS increase and glutathione depletion are critical to GA cytotoxicity. Thus, oxidative stress in conjunction with glutathione depletion plays a role in the cytotoxicities of both GA and MEN.

Effect of GA on activation and stabilization of Hsp90 client proteins

To determine the effect of GA-induced stress on expression and activation of the survival kinases Akt and ERK1/2, cell lysates were separated and immunoblotted using specific antibodies to their phosphorylated forms. ERK1/2 phosphorylation strongly increased in MEN-treated cells at 1h and 6h, while increasing moderately with GA at 1h, and more significantly at 6h. Akt phosphorylation briefly increased at 1h in GA-treated cells. Total expression of these kinases was unaltered, suggesting Akt and ERK1/2 activation may occur as a result of early stress response prior to the onset of cell death (Fig 4A). ERK1/2 phosphorylation increase was sustained at 6h while Akt phosphorylation and total expression were reduced (Fig 4B). Protein expression further decreased at 24h, as the effect of GA and of MEN may become less specific, also affecting proteins that may not bind to the Hsp90 complex, such as β -actin and ERK1 (Fig 4C). Alternatively, these decreases may reflect extensive cell death. Antioxidant treatment with NAC inhibited both GA- and MEN-induced phosphorylation increases and total protein

degradation, suggesting that oxidative stress plays a role in GA- and MEN-induced cellular stress (Fig 4). These data suggest that GA-induced quinone stress increases protein oxidation that may enhance protein ubiquitination and degradation. Indeed, ubiquitin immunoblots of lysates show that both GA and MEN induce total protein ubiquitination which was inhibited by NAC (Fig 5).

Effect of GA on Hsp90 binding to client proteins

To determine whether oxidative stress plays a role in GA-induced disruption of the Hsp90 complex, Hsp90 was immunoprecipitated from lysates of cells treated with GA, MEN, or DMSO, with or without NAC pre-treatment. Immunoblots of Hsp90 immunoprecipitates show that 1h GA treatment decreased Cdc37 and Raf-1 binding to Hsp90, while MEN did not affect their binding (Fig 6). However, NAC, shown to inhibit ROS formation at this time point (Fig 2A), did not reverse the 1h GA-induced disruption of Hsp90 binding to Raf-1 and Cdc37 (Fig 6). Thus, GA-induced disruption of Hsp90 binding appears independent from the effect of GA on cellular oxidative status at this early time point. These findings were confirmed by immunoprecipitation of Hsp90-binding proteins, followed by immunoblotting for Hsp90 and Hsp90-binding proteins (data not shown). Longer GA treatments increased dissociation of the Hsp90 complex, while MEN still failed to affect protein binding (Fig 7). Therefore, while sufficient to induce cell death starting at 6h, oxidative stress alone, as induced by MEN, does not disrupt Hsp90 complexes. NAC did not prevent GA-induced early protein dissociation, but allowed re-association of proteins with Hsp90 starting at 6h, completely reversing protein dissociation by 24h. These data suggest that oxidative stress does not dissociate the Hsp90 complex. In addition, although both GA and MEN generate ROS that are required for cell death, their mechanisms of cytotoxicity differ.

Role of the ubiquitin-dependent proteasomal degradation pathway in GA- and MEN-induced protein degradation

Both GA and MEN increased total protein ubiquitination at 1h and 6h, demonstrating that the onset of protein ubiquitination occurs prior to the decrease in protein expression (Fig 5). NAC inhibited these increases in ubiquitination (Fig 5), suggesting that quinone-induced oxidative stress increases protein ubiquitination.

To determine whether GA-induced ROS increase occurs upstream or downstream of proteasomal degradation of proteins, DCF fluorescence was measured in cells treated with the proteasome inhibitor MG-132 and exposed to GA or MEN. MG-132 increased accumulation of ubiquitinated proteins (Fig 9A, 10A), but did not significantly affect expression of β -actin or of the cytosolic superoxide dismutase 1 (SOD-1; Fig 9B, 10A, 10B), suggesting that degradation of ubiquitinated proteins was indeed inhibited. However, while MG-132 inhibited MEN-induced increases in ROS (Fig 8), protein degradation (Fig 10B), and cell death (Fig 11), MG-132 did not alter GA-induced ROS (Fig 8), or the decreases in expression of Hsp90, Cdc37, Akt, Raf-1, ERK1, and β -actin (Fig 10B), or its cytotoxicity (Fig 11). Therefore, GA-induced degradation of Hsp90-binding proteins is not dependent on the proteasomal degradation pathway and alternative pathways may be involved. A potential degradation pathway, the lysosomal pathway, can be inhibited by Bafilomycin A1 (BAF). BAF prevents intralysosomal acidification, thereby affecting degradation of lysosomal content. Increasing doses of up to 750 nM BAF failed to prevent GA-induced degradation of Hsp90-binding proteins (data not shown). These findings suggest again that the mechanism of cytotoxicity induced by these two compounds differ. MEN induction of oxidative stress results in proteasomal protein degradation, increasing ROS and cytotoxicity without disrupting Hsp90 binding. In contrast, GA cytotoxicity involves disruption of Hsp90 binding and requires oxidative stress, but does not require proteasomal or lysosomal activity to induce protein degradation and cell death.

Discussion

The protein interactions of Hsp90 complexes have been implicated in survival of various cell types. Disruption of Hsp90 binding to Akt induces apoptosis [14]. Hsp90 has been previously shown to be required for stability and activation of the survival kinase Akt [13–15] and of additional Hsp90-binding proteins, such as Raf-1 and the kinase co-chaperone Cdc37 that may also regulate cell survival. These Hsp90 complexes are abundantly expressed in cancer cells. Therefore, pharmacological inhibitors of Hsp90 binding may simultaneously impact several oncogenic signaling pathways and have been developed for the treatment of a variety of cancers. Disruption of the Hsp90 complex by these inhibitors destabilizes the Hsp90-binding proteins, inducing cell death. However these compounds are also highly toxic to surrounding cells. Elucidating the mechanism of Hsp90 inhibitor cytotoxicity may allow development of therapeutic strategies to improve targeting of cancer cells.

To study the mechanism of toxicity of these compounds, the classical Hsp90 inhibitor Geldanamycin was used to determine the role of Hsp90 binding and stabilization of Akt, Raf-1, and Cdc37 in the regulation of PC-12 cell survival. GA induces PC-12 cell death, suggesting a role for Hsp90 interactions in survival of these cells. Our findings show that GA cytotoxicity requires GA-induced oxidative stress and glutathione depletion, correlating with decreased expression of Hsp90-binding proteins and, at a later time point, with decreased expression of additional non-Hsp90 binding proteins (Fig 12).

GA cytotoxicity has been attributed to its disruption of Hsp90 binding. However, GA is also a source of oxidant radicals [25,26] through its quinone moiety, and has been shown to deplete GSH cellular pools. Indeed, GA ability to bind GSH and form stable thiol adducts at physiological pH and concentration contributes to decreased GSH availability [27]. To examine the role of oxidative stress in the drug's cytotoxicity and in the disruption of Hsp90 binding we used an antioxidant strategy and compared the effects of GA to those of MEN, a classical quinone that can deplete cellular GSH but does not interact with Hsp90. Our comprehensive study of Hsp90 binding demonstrates for the first time a critical role for oxidative stress in conjunction with GSH depletion in GA cytotoxicity, independently from its disruption of the Hsp90 complex. We established that cytotoxic, MEN-induced quinone stress alone was insufficient to dissociate the Hsp90 complex. While MEN indiscriminately targeted all cells, we uncovered a dual mechanism for GA-induced cell death that may allow improved targeting of cancer cells. GA induced early disruption of the Hsp90 complex and oxidation of proteins, followed by their degradation, resulting in cytotoxicity. While inhibition of ROS formation with Mn TBAP did not suffice to rescue GA-treated cells, the GSH precursor NAC removed ROS and restored GSH levels, inhibiting GA-induced cell death and restoring protein binding to the complex. Indeed, MnTBAP has been shown to work as a superoxide dismutase mimetic, scavenging superoxide, hydrogen peroxide and peroxynitrite [28] without affecting GSH levels. In contrast, NAC may restore GSH that may bind to Hsp90 and displace GA binding, allowing re-association of Hsp90 client proteins. Indeed, addition of GSH has been reported to increase cancer cells resistance to benzoquinone ansamycin compounds [25,32].

Cancer cell expression of Hsp90 and its binding proteins Akt, Raf-1, and Cdc37 is significantly higher compared to normal cells. Therefore, the cytotoxicity of GA and its derivatives can be modulated using antioxidant compounds to disrupt Hsp90 binding can and target Hsp90 and its binding proteins in Hsp90 over-expressing cancer cells, while potentially minimizing toxicity to normal cells. However, this strategy needs to be modulated to fit the variability of cancer cell tolerance to oxidative stress. Upregulation of Hsp27 which positively regulates GSH levels, is associated with the resistance of cancer cells to GA cytotoxicity, and depleting GSH restores cell vulnerability [32]. Additionally, GSH levels regulate cancer cell resistance to radiation therapy [31]. Although GA was initially discovered as an Hsp90-targeting drug,

GA and MEN are among several anticancer drugs also known to decrease GSH [27,29]. We considered whether Hsp90 binding regulates cell survival by regulating cellular oxidative stress. Nardai *et al.* propose a role for Hsp90 in regulating the redox state of cells by reducing proteins, and show that this function of Hsp90 is blocked by sulfhydryl reagents that may target the chaperone's cysteine residues [33]. However, this study by Nardai *et al.* did not examine whether this novel putative function of Hsp90 is critical to cell survival. GA and MEN may act as sulfhydryl reagents by oxidizing critical cysteine residues of Hsp90 and may thereby inhibit its ability to regulate cellular oxidative stress, resulting in cytotoxicity. Thus by targeting cells highly expressing Hsp90 and by modulating cellular oxidant status, GA may increase cytotoxicity towards cancer cells rather than normal cells.

The degradation of dissociated oxidized Hsp90-binding proteins may play a significant role in benzoquinone ansamycin compounds cytotoxicity. Hsp90 inhibitors have been reported to induce ubiquitination and proteasomal degradation of several Hsp90-binding proteins [12,34, 35]. However, these studies did not examine the effects of proteasomal inhibition on cell viability in presence or absence of Hsp90 inhibition. Furthermore, Hsp90 inhibition by GA has been shown to induce proteasome-dependent internalization and subsequent lysosomal degradation of the receptor tyrosine kinase ErbB2, an Hsp90-binding protein localized at the plasma membrane (Fig 12; [36]). In addition, Hsp90 inhibition by GA was shown to increase chaperone mediated autophagy (CMA) that targets proteins for lysosomal degradation [37]. Recently, GA has been shown to disrupt Hsp90 binding to I κ B kinases as well as NF κ B-inducing kinase, targeting them to a non-proteasomal autophagic degradation [38,39]. These data agree with our findings that a non-proteasomal degradation pathway degrades dissociated Hsp90-binding proteins. However, oxidative stress may induce CMA of oxidized proteins [40], and the role of GA-induced oxidative stress in CMA was not examined in these studies.

Our results show increased protein ubiquitination in GA- and MEN-treated PC-12 cells. Proteasomal inhibition prevented protein degradation ROS formation and cell death in MEN-treated cells, but not in GA-treated cells. While both compounds induce oxidative stress and GSH depletion resulting in increased protein ubiquitination, MEN did not dissociate proteins from Hsp90. Despite increased accumulation of ubiquitinated proteins in GA-treated cells pretreated with MG-132, dissociated Hsp90-binding proteins still degraded. MG-132 inhibition of ROS and cell death in MEN-treated cells was surprising since we expected proteasomal inhibition to cause accumulation of ubiquitinated proteins and cell toxicity. However a recent study reported that low dose of MG-132 can protect nerve cells from oxidative injury [40]. These findings suggest that disruption of Hsp90 binding by GA induces proteasome-independent degradation of Hsp90-binding proteins, while the non-specific quinone stress of MEN induces proteasomal degradation. Therefore, Hsp90 binding may potentially affect protein degradation pathways, targeting proteins to proteasomal degradation when proteins are bound to Hsp90, while proteins dissociated from Hsp90 may be degraded by an alternative pathway.

To examine potential alternative degradation pathways, we used the lysosomal inhibitor Bafilomycin A₁ (BAF), known to inhibit lysosome acidification in PC-12 cells [40]. However, BAF did not alter GA-induced degradation of Akt and Raf-1, suggesting that these proteins are not degraded in the lysosomes upon disruption of Hsp90 binding. Additional degradation pathways that may be implicated include protease-dependent degradation pathways, such as caspase and calpain cysteine proteases.

Caspase-3 has been identified as an Hsp90 binding protein, suggesting that Hsp90 binding may also regulate caspase activation [41]. GA has been reported to induce caspase-dependent apoptosis in human glioma cells, correlating with decreased expression of phosphorylated Akt [42]. Furthermore, Hsp90 binds to various caspase substrates proteins and regulates their

proteasomal degradation [43], suggesting that Hsp90 may function as a scaffold for caspases and their substrates, or that Hsp90 may prevent cleavage of caspase substrates by sequestering inactive caspases.

Similarly to caspases, calpains are involved in protein degradation in apoptotic and in necrotic cells and have been implicated in several neuropathologies [44–48]. Hsp90 has been reported to protect several kinases and enzymes from calpain-dependent degradation. Hsp90 binding to both calpain and nitric oxide synthase (NOS) protects NOS from calpain-dependent degradation in the rat brain [49,50]. Calpain degrades some proteins independently of the proteasome and processes other proteins for proteasomal degradation [51,52]. Future studies will determine whether caspase-dependent and or calpain-dependent proteolytic degradation are implicated in Hsp90 dissociated proteins degradation.

In conclusion, Hsp90 binding may regulate cellular survival of PC-12 cells by regulating the degradation of proteins (Fig 12). While demonstrating a role for oxidative stress in GA-induced cytotoxicity, the current study did not allow us to dissociate entirely between the two main effects of GA, dissociation of the Hsp90 complex and quinone stress. However, in an elegant study using anti-Hsp90 ribozymes, Sreedhar et al. demonstrated that inhibition of Hsp90 chaperone function alone can compromise cellular integrity and increase vulnerability to various types of injury, even in presence of NAC [25]. Therefore, inhibition of Hsp90 chaperone function by GA may increase cancer cells vulnerability to oxidative stress. In PC-12 cells, Hsp90 binding is insufficient to protect from severe oxidative stress when GSH is depleted, such as in MEN-induced injury. However, Hsp90 binding may protect against physiological oxidative stress. Our findings suggest that, GA can be targeted against most cancer cells as these cells express higher levels of Hsp90 and Hsp90-binding proteins, and increase their susceptibility to oxidative stress and other insults. A deeper understanding of the chaperone functions of Hsp90 and of the mechanisms of cytotoxicity of the benzoquinone ansamycin Hsp90 inhibitors may lead to the development of novel therapies for the treatment of cancers and neurological disorders.

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List of abbreviations

Hsp90	Heat shock protein 90
GA	Geldanamycin
MEN	Menadione
ROS	Reactive oxygen species
NAC	N-acetyl cysteine
MTT	3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide

DCF	2',7'-dichlorofluorescein
BAF	Bafilomycin A ₁
HRP	Horseradish peroxidase
GSH	Glutathione
CMA	Chaperone mediated autophagy
SOD-1	Superoxide dismutase 1

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Figure 1. Time course of GA-induced cell death in PC-12 cells

Significant cell death occurred at 6h with 10 μ M Geldanamycin (GA; open bars) and was enhanced at 20 μ M GA (black bars). Cell death was assessed by MTT assay and expressed as percent of vehicle treated cells at the same time point. * = $p < .01$ for 10 μ M GA vs. 20 μ M GA at same time point; † = $p < .05$ for same dose of GA at different time points; Data are expressed as mean $\pm$ SD ($n \geq 5$).

Figure 2. Time course of GA-induced ROS in PC-12 cells

20 μ M GA induced ROS production, measured by DCF or DHE assay, that was inhibited by NAC but not by MnTBAP. DMSO was used as a control and 20 μ M menadione as a positive control. (A-B) 1h, (C) 6h, (D) 24h. C = Control, MnT = MnTBAP; N = NAC; D = DMSO, M = Menadione, GA = Geldanamycin. * = $p < .001$ or ¶ = $p < .05$ for Control + DMSO vs. other conditions; M = $p < .05$ for Control + GA vs. Control + MEN; Data are expressed as mean $\pm$ SD (n > 5).

Figure 3. Effect of oxidative stress on GA-induced cell death

20 μ M GA and MEN induced cell death, assessed by Trypan blue exclusion assay, was inhibited by NAC at 1h (A), 6h (B) or 24h (C) but not by MnTBAP at 16h (D). C = Control, N = NAC; Treatments: D = DMSO, M = Menadione, GA = Geldanamycin, MnT = MnTBAP * = $p < .05$ for Control + 6h DMSO vs. other 6h treated cells; * = $p < .05$ for Control + DMSO vs. other treatments; ¶ = $p < .0001$ for DMSO vs. GA. Data are expressed as mean $\pm$ SD ($n > 5$).

Figure 4. Effect of GA on protein expression: Role of oxidative stress

NAC inhibited 20 μ M GA- and MEN-induced decreases in the expression of Hsp90-binding proteins and other proteins at 1h (A), 6h (B), or 24h (C). C = Control, N = NAC, M = MEN, GA = Geldanamycin ($n \geq 3$).

Figure 5. Effect of GA on protein ubiquitination: Role of oxidative stress

NAC inhibited 20 μ M GA- and MEN-induced protein ubiquitination at 1h (A) and 6h (B). C = Control, N = NAC, M = Menadione, GA = Geldanamycin ($n \geq 3$).

Figure 6. Effect of 1h GA on Hsp90 protein binding in PC-12 cells: Role of oxidative stress
 Representative immunoblots of Hsp90 immunoprecipitates show that at 1h of treatment, 20 μ M GA-induced disruption of Cdc37 and of Raf-1 binding to Hsp90 was not altered by NAC, while MEN did not disrupt Hsp90 binding. C = Control, N = NAC, D = DMSO, M = Menadione, GA = Geldanamycin. IgG = non-immune isotype control antibody (n = 3).

Figure 7. Effect of prolonged GA treatment on Hsp90 protein binding in PC-12 cells. Role of oxidative stress

Representative immunoblots of Hsp90 immunoprecipitates show that 6h and 24h GA but not MEN disrupts Cdc37 and Raf-1 binding to Hsp90. At 24h, Cdc37 and Raf-1 binding is restored by NAC treatment. C = Control, N = NAC, D = DMSO, M = Menadione, GA = Geldanamycin. IgG = non-immune isotype control antibody (n $\geq$ 3).

Figure 8. Role of the proteasomal pathway in GA-induced oxidative stress

Proteasome inhibition by MG-132 inhibits 20 μ M MEN-induced ROS but not 20 μ M GA-induced ROS, as shown by DCF fluorescence assay, at 1h (A), 9h (B), and 27h of treatment (C). D = DMSO, MG = MG-132, M = Menadione, GA = Geldanamycin. * = p < .05 for DMSO + MEN vs. MG-132 + MEN at the same time point; Data are expressed as mean $\pm$ SD (n $\geq$ 5).

Figure 9. Role of the proteasomal pathway in GA-induced protein ubiquitination

Representative immunoblots show that MG-132 and a short 20 μ M GA treatment increase ubiquitination of proteins without altering the expression of the cytosolic antioxidant superoxide dismutase 1 (SOD-1) and β -actin. D = DMSO, GA = Geldanamycin, MG = MG-132 (n = 3).

Figure 10. Role of the proteasomal pathway in prolonged GA-induced protein ubiquitination
 Representative immunoblots show that **(A)** MG-132 increased proteins ubiquitination in the presence of 20 μ M MEN and GA, without increasing superoxide dismutase 1 (SOD-1) expression. **(B)** MG-132 inhibited MEN-induced decreases in Hsp90, Cdc37, Akt, Raf-1, ERK1, and β -actin expression, but did not inhibit GA-induced decreases in expression of proteins. D = DMSO, MG = MG-132, M = Menadione, GA = Geldanamycin (n = 3).

Figure 11. Effect of proteasomal inhibition on cell survival to GA and to MEN
 Proteasomal inhibition by MG-132 decreased MEN but not GA cytotoxicity at 1h (A), 9h (B), and 27h (C). Cell survival was measured by MTT assay and expressed as percent DMSO control. D = DMSO, MG = MG-132, M = Menadione, GA = Geldanamycin. * = $p < .01$ for DMSO-pre-treated vs. MG-132-pre-treated for the same GA, DMSO, or MEN exposure, † = $p < .05$ for exposed cells vs. DMSO + DMSO treatment. Data are expressed as mean $\pm$ SD ($n \geq 5$).

Figure 12.

Schematic representation of the effects of Geldanamycin (GA) on the Hsp90 complex and the role of oxidative stress in GA-induced cell death.