

**A novel Hsp90 inhibitor activates compensatory heat shock protein responses and
autophagy and alleviates mutant A53T α -synuclein toxicity**

**Rui Xiong, Wenbo Zhou, David Siegel, Russell R. A. Kitson, Curt R. Freed, Christopher J.
Moody and David Ross**

Department of Pharmaceutical Sciences, Skaggs School of Pharmacy and Pharmaceutical
Sciences (*R.X, D.S, D.R*), Department of Medicine, Division of Clinical Pharmacology and
Toxicology (*W.Z, C.R.F*), University of Colorado Anschutz Medical Campus, Aurora, CO, USA;
School of Chemistry, University of Nottingham, Nottingham, UK (*R.R.A.K., C.J.M.*)

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To whom correspondence should be addressed: David Ross, Department of Pharmaceutical Sciences, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of Colorado Anschutz Medical Campus, 12850 E. Montview Blvd, Aurora, CO 80045. Tel.: 303-724-7265; Fax: 303-724-7266; E-mail: David.Ross@ucdenver.edu

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Abbreviations:

17-AAG, 17-allylamino-17-demethoxygeldanamycin;

17-DMAG, 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin;

19BQA, 19-substituted benzoquinone ansamycin;

19-DMA-GA, 19-dimethylamino-geldanamycin;

19-HM-GA, 19-hydroxymethyl-geldanamycin;

19-Me-GA, 19-methyl-geldanamycin;

19-Naphthyl-GA, 19-naphthyl-geldanamycin;

19-Ph-GA, 19-phenyl-geldanamycin;

19-Ph-Mor-GA, 19-(4-morpholinyl-phenyl)-geldanamycin;
19-Ph-OMe-GA, 19-(4-methoxy-phenyl)-geldanamycin;
19-Ph-Ph-GA, 19-(4-phenyl-phenyl)-geldanamycin;
ALP, autophagy-lysosome pathway;
BQA, benzoquinone ansamycins;
CQ, chloroquine;
eIF2 α , eukaryotic translation initiation factor 2 alpha;
GA, geldanamycin;
HSPs, heat shock proteins;
Hsp70, heat shock protein 70;
Hsp27, heat shock protein 27;
HW α -Syn, higher molecular weight α -Syn;
LC3, autophagy related microtubule-associated protein 1 light chain 3;
mTOR, mammalian target of rapamycin;
 α -Syn, α -synuclein;
p70S6K, the p70 ribosomal S6 kinase;
PD, Parkinson's disease;
PI, propidium iodide;
ROS, reactive oxygen species;
TFEB, transcriptional factor EB;
UPR, unfolded protein response;
UPS, ubiquitin-proteasome system.

ABSTRACT

A potential cause of neurodegenerative diseases including Parkinson's disease (PD) is protein misfolding and aggregation that in turn leads to neurotoxicity. Targeting Hsp90 is an attractive strategy to halt neurodegenerative diseases, and benzoquinone ansamycin (BQA) Hsp90 inhibitors such as geldanamycin (GA) and 17-allylamino-17-demethoxygeldanamycin (17-AAG) have been shown to be beneficial in mutant A53T α -synuclein PD models. However, current BQA inhibitors result in off-target toxicities via redox cycling and/or arylation of nucleophiles at the C19 position. We developed novel 19-substituted BQA (19BQA) as a means to prevent arylation. In this study, our data demonstrated that 19-phenyl-GA, a lead 19BQA in the GA series, was redox stable and exhibited little toxicity relative to its parent quinone GA in human dopaminergic SH-SY5Y cells as examined by oxygen consumption, trypan blue, MTT and apoptosis assays. Meanwhile, 19-phenyl-GA retained the ability to induce autophagy and potentially protective heat shock proteins (HSPs) such as Hsp70 and Hsp27. We found that transduction of A53T, but not wild type (WT) α -synuclein induced toxicity in SH-SY5Y cells. 19-phenyl-GA decreased oligomer formation and toxicity of A53T α -synuclein in transduced cells. Mechanistic studies indicated that mTOR/p70S6K signaling was activated by A53T but not WT α -synuclein and 19-phenyl-GA decreased mTOR activation that may be associated with A53T α -synuclein toxicity. In summary, our results indicate that 19BQAs such as 19-phenyl-GA may provide a means to modulate protein-handling systems including HSPs and autophagy thereby reducing the aggregation and toxicity of proteins such as mutant A53T α -synuclein.

Introduction

Hsp90 is a crucial molecular chaperone that functions to correctly fold client protein, thereby preventing them from degradation by the ubiquitin-proteasome system (UPS) (Neckers, 2002). More than 200 client proteins of Hsp90 have been identified including a range of prosurvival proteins (also known as oncoproteins) such as Raf-1, Akt, p53 and cdk4 (Kamal and Burrows, 2004) as well as neural proteins including polyglutamine-expanded mutant androgen receptor (Tokui *et al.*, 2009) and leucine-rich repeat kinase 2 (LRRK2) (Hurtado-Lorenzo and Anand, 2008).

One of the major causes of neurodegenerative diseases including Parkinson's Disease (PD) is the accumulation of misfolded protein aggregates that in turn leads to toxicity and loss of neurons (Olzmann *et al.*, 2008; Olanow and McNaught, 2011). In the brain, α -synuclein (α -Syn) is highly abundant and plays a key role in the modulation of synaptic activity. α -Syn is also the main component of Lewy bodies (LW) and Lewy neurites (LN) in the PD brain, and mutations in the gene encoding α -Syn (SNCA gene) have been implicated in the early onset of familial forms of PD (Lashuel *et al.*, 2013). A53T α -Syn is one of the mutant forms of α -Syn that has been intensively studied. Despite accumulating evidence for mutant A53T α -Syn in the pathogenesis of PD its role has not been fully elucidated. However, it is believed that the toxicity of A53T α -Syn is associated with formation of pre-fibrillar α -Syn oligomers (Kalia *et al.*, 2013).

Recent studies suggest that inhibition of Hsp90 could be beneficial for neurodegenerative diseases (McLean *et al.*, 2004; Waza *et al.*, 2005; Tokui *et al.*, 2009; Baldo *et al.*, 2012). Pharmacologic inhibition of Hsp90 has been shown to be useful in neurodegenerative disease models (Gallo, 2006; Luo *et al.*, 2010). There are at least three potential protective mechanisms associated with inhibition of Hsp90; 1) Hsp90 inhibition leads to activation of heat shock factor

1 (Hsf-1) that further induces a range of small heat shock proteins (including Hsp70 and Hsp27) which assist in appropriate folding of misfolded proteins (Dimant *et al.*, 2012); 2) Inhibition of Hsp90 leads to the degradation of mutant neuronal client proteins such as polyglutamine-expanded mutant AR and leucine-rich repeat 2 (LRRK2) which reduces the load of toxic Hsp90 client proteins (Waza *et al.*, 2005; Tokui *et al.*, 2009; Taipale *et al.*, 2010; Baldo *et al.*, 2012) and (3) Hsp90 inhibitors including 17-AAG have been shown to induce protective autophagy that led to degradation of mutant and misfolded protein aggregates (Riedel *et al.*, 2010). The benzoquinone ansamycin (BQA) Hsp90 inhibitors GA, 17-AAG and 17-DMAG have been shown to induce a robust compensatory heat shock response, and amelioration of protein aggregation and toxicity. Specifically with respect to PD, either GA or 17-AAG can inhibit α -synuclein aggregation and toxicity in cell, fly and yeast models (Waza *et al.*, 2005; Tokui *et al.*, 2009; Putcha *et al.*, 2010; Baldo *et al.*, 2012).

However, current BQAs inhibitors were relatively hepatotoxic in animal models and their off-target toxicities were mediated via redox cycling and arylation of nucleophiles at the C19 position of the benzoquinone ring (Kamal and Burrows, 2004; Cysyk *et al.*, 2006; Guo *et al.*, 2008). We developed novel 19-substituted BQAs (19BQAs) as a means to prevent arylation and have validated this approach by using model thiols (Kitson *et al.*, 2013; Chang *et al.*, 2014). This study was designed to evaluate the use of novel 19BQAs as potential therapeutic agents for PD. We established a transient A53T α -Syn overexpression model and used 19-Ph-GA as proof of principle that i) 19BQAs had reduced toxicity relative to GA in SH-SY5Y cells and ii) were able to induce compensatory changes in protein handling to ameliorate A53T α -Syn oligomer formation and toxicity.

Materials and Methods

Materials and Antibodies. 17-AAG and GA were purchased from LC Laboratories (Woburn, MA); 19-phenyl-GA-(19-Ph-GA), 19-methyl-GA (19-Me-GA), 19-(4-phenyl-phenyl)-GA (19-Ph-Ph-GA), 19-(4-morpholinyl-phenyl)-GA (19-Ph-Mor-GA), 19-(4-methoxy-phenyl)-GA (19-Ph-OMe-GA), 19-dimethylamino-GA (19-DMA-GA), 19-hydroxymethyl-GA (19-HM-GA) were synthesized as described previously (Kitson *et al.*, 2013; Kitson and Moody, 2013). NADH, DAPI, chloroquine diphosphate, ATP disodium salt, digitonin, phenylmethylsulfonyl fluoride (PMSF), bovine serum albumin and mouse monoclonal anti- β -actin (A5441) antibody were obtained from Sigma Chemical (St. Louis, MO, USA). Dithiothreitol was purchased from Fisher Scientific (Pittsburgh, PA, USA). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] was obtained from Research Products International (Mount Prospect, IL, USA). Mouse anti- α -synuclein (610786) antibody was from BD transduction laboratories (San Jose, CA, USA). Mouse monoclonal anti-Hsp27 (ADI-SPA-800), anti-Hsp70 (ADI-SPA-810) antibodies and rabbit polyclonal anti-p62 (BML-PW9860) antibody were purchased from Enzo (Farmingdale, NY, USA). FITC / annexin V (640906) and annexin V binding buffer (422201) were purchased from BioLegend (San Diego, CA, USA). The proteasome substrate Suc-Leu-Leu-Val-Tyr-AMC was purchased from Bachem (Torrance, CA, USA). Rabbit polyclonal anti-LC3 (NB100-2331 and NB600-1384) antibody was from Novus (Littleton, CO, USA). Rabbit polyclonal anti-phospho-mTOR (Ser2448, #2971), anti-mTOR (#2972), anti-phospho-P70S6K (Thr389, #9205), anti-P70S6K (#9202), anti-phospho-eIF2 α (Ser51, #9721) and anti-eIF2 α (#9722) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

Cell culture and treatment. SH-SY5Y cells were purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM/F-12 medium (Cellgro, Manassas,

VA, USA) containing 10% (v/v) fetal bovine serum, 2mM glutamine (ATCC, Manassas, VA, USA) 100 units/ml penicillin and 100 µg/ml streptomycin (Cellgro, Manassas, VA, USA) in a humidified atmosphere of 5% CO₂ at 37°C.

Adenoviral transient transduction. Adenoviruses expressing either A53T mutated α -synuclein (A53T α -Syn), wild-type α -synuclein (WT α -Syn) or green fluorescent protein (GFP) were constructed and produced in two-step Ad-Easy systems (Zhou *et al.*, 2002). Adenoviruses were mixed with culture medium and incubated with cells at a concentration of 100 to 400 plaque forming units (pfu)/cell as described previously (Zhou *et al.*, 2002; 2011).

MTT growth inhibition assay. Drug induced toxicity was determined using the MTT assay. SH-SY5Y cells were seeded at 5000 cells per well in 96-well plates or 40000 cells per well in 48-well plates (in triplicate). After 24h the cells were incubated with adenoviruses expressing either A53T mutated α -synuclein (A53T α -Syn) or wild-type α -synuclein (WT α -Syn) in complete medium in 48-well plates. One day after adenovirus transduction cells were exposed to 19-substituted-benzoquinone ansamycins (19BQAs) at varying concentrations for 48h after which, the medium were removed and replaced with complete medium containing MTT (0.4mg/ml). The MTT containing medium was removed after 2h incubation and the MTT formazan product was extracted from cells with 200µl of dimethyl sulfoxide. The optical density of the extract was determined at 550nm with a microplate reader. IC₅₀ values were defined as the concentration of drugs that resulted in a 50% reduction in cell density compared to DMSO treated controls.

Trypan blue exclusion assay. Cells were seeded at 0.5×10^6 cells into 60 mm \times 15 mm tissue culture dishes in 3 ml of complete medium and allowed to grow for 24h. Following treatment with the indicated compound for 24h, cell pellets containing both floating and attached

cells were collected and then resuspended in 1ml PBS. To assess cell viability the cell suspension (10 μ l) was stained with 10 μ l trypan blue dye (0.4% w/v in PBS). The number of viable cells was determined using a Life Technologies Countess™ automated cell counter immediately after staining.

Apoptosis assay. Apoptosis was determined by flow cytometry using FITC-conjugated anti-annexin V and propidium iodide (PI) as previously described (Xiong *et al.*, 2013). After the indicated treatments, cell pellets including both the floating and attached cells were collected and resuspended in 500 μ l of annexin V binding buffer and incubated with anti-annexin V antibody (2 μ l) and PI (0.7 μ l of 100 μ g/ml stock) for 10 min at 37°C in the dark. Samples were kept on ice, and then analyzed using a BD Biosciences FACS Calibur Flow Cytometer (San Jose, CA, USA). The fluorescence was measured at 530nm (FL1, FITC) and PI at above 600nm (FL2), and the data was acquired and analyzed using Cellquest software (Becton-Dickenson, Mountainview, CA, USA).

Proteasomal activity assay. Proteasomal activity was determined by measuring the fluorescence of cleaved proteasome substrate Suc-Leu-Leu-Val-Tyr-AMC (chymotrypsin-like activity) as described previously (Kisselev and Goldberg, 2005; Xiong *et al.*, 2013; 2014). Briefly, after the indicated treatments, the medium was removed and the cells were washed with PBS (3ml) three times, and then lysed with proteasomal activity assay buffer and centrifuged at 10,000g for 14min at 4°C. The supernatants were collected and protein concentrations were determined using the method of Lowry (Lowry *et al.*, 1951). To assay proteasome activity cell lysate (20 μ g) was added to 100 μ l of proteasome activity assay buffer (50mM Tris-HCl, pH 7.5, 250mM sucrose, 1mM dithiothreitol, 5mM MgCl₂, 2mM ATP, 0.5mM EDTA and 0.025% (w/v) digitonin) followed by the addition of 100 μ M proteasome substrate Suc-Leu-Leu-Val-Tyr-AMC.

After incubation at 37°C for 30 min, 40µl samples were transferred to a 96-well plate and the fluorescence of liberated 7-amino-4-methylcoumarin (Ex: 380nm; Em: 460nm) was measured in duplicate samples.

Immunoblot analysis. Cells were seeded at 400,000 cells per well in 6-well plate. Following the indicated treatments cells were washed with PBS and then attached cells were collected and lysed with RIPA buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 0.1% (w/v) SDS, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate) supplemented with protease inhibitors (Complete, Mini Protease Inhibitor Cocktail, Roche, Germany), PMSF (2mM) and phosphatase inhibitors (Sigma Chemical, USA). Cells were sonicated on ice and then centrifuged at 10,000g for 14 min at 4°C. The supernatants were collected and protein concentrations were measured using the method of Lowry (Lowry *et al.*, 1951). Proteins were diluted in 2X Laemmli SDS sample buffer and heated to 70°C for 5 min, and then separated on a 12% SDS-PAGE precast minigel (BioRad Laboratories, Hercules, CA, USA). A 7.5% SDS-PAGE precast minigel was used to separate the high molecular weight oligomers. Proteins were transferred to polyvinylidene fluoride membranes in 25mM Tris, 192mM glycine containing 20% (v/v) methanol at 4°C. Membranes were incubated in blocking buffer (10mM Tris-HCl, pH 8.0, 150mM NaCl, 0.2% (v/v) Tween-20 (TBST) containing 5% (w/v) non-fat dry milk) for 1h, and then incubated with primary antibodies overnight at 4°C. Membranes were washed every 10min in TBST for 1h and then probed with horseradish peroxidase-conjugated goat anti-rabbit or horseradish peroxidase-conjugated goat anti-mouse IgG (1:5000; Jackson Immunoresearch Labs, West Grove, PA, USA) for 30min at room temperature. Membranes were washed three times in TBST and protein bands were visualized using enhanced chemiluminescence (Thermo Scientific, USA). Adobe Photoshop CS6 was used to quantitate immunoblots from three independent experiments.

Oxygen consumption assay. The ability of 19BQAs to undergo redox cycling in SH-SY5Y cells was determined using an airtight Clark electrode (Yellow Springs Instrument Company, Yellow Springs OH, USA). For these studies 5 million cells were suspended in 3ml of complete cell culture medium at 37°C in the presence or absence of compounds and the rate of oxygen consumption was measured over 10min Dissolved oxygen concentrations were adjusted for altitude (5280 feet) and temperature (37°C).

Statistical analysis. All experiments were repeated at least three times, and the values were expressed as means \pm standard deviation (SD). The statistical significance was determined using Prism 6.0 software (GraphPad Software, San Diego, CA). One-way analysis of variance (ANOVA) followed by the Tukey or Dunnet's multiple comparison tests was used.

Results

Decreased toxicity of 19BQAs relative to their parent quinone geldanamycin (GA) in SH-SY5Y cells. The synthesis of 19-substituted-benzoquinone ansamycins (19BQAs, Fig. 1) was described previously (Kitson *et al.*, 2013) . We first examined the potential toxicity of 19BQAs in human dopaminergic SH-SY5Y (5Y) cells using a short term MTT test as an initial screen. As shown in Figure 2A, GA induced a significant loss in cell viability at low concentration (IC_{50} 287nM), compared to 19-Ph-GA (IC_{50} >10 μ M). In addition, we found that 19BQAs were also less toxic than 17-DMAG (IC_{50} 6.8 μ M). Overall, these results using this initial screen indicated that 19BQAs induced substantially decreased toxicity relative to GA and 17-DMAG.

The relatively low toxicity of 19-Ph-GA was then confirmed using the trypan blue exclusion assay and flow cytometry using annexin V and propidium iodide (PI) double staining for apoptosis. As shown in Figure 2B, no significant toxicity could be detected following treatment with 19-Ph-GA (250nM) for 24h while exposure to GA at the equivalent concentration resulted in over 30% of cell death and significant morphological changes in 5Y cells (Supplementary Fig. 1). Significant induction of apoptosis (Fig. 2C) was also observed after 24h treatment with a higher concentration (5 μ M) of GA while exposure to 19-Ph-GA (5 μ M) did not increase numbers of apoptotic cells.

19-substitution prevents the ability of BQAs to induce toxicity via arylation of cellular nucleophiles (Chang *et al.*, 2014). An additional concern with quinone based drugs is their ability to undergo redox cycling and generate reactive oxygen species (ROS) which in turn results in off-target toxicities. To determine whether 19BQAs undergo appreciable redox cycling, we measured the rates of oxygen consumption in 5Y cells in the absence and presence of GA or

19-Ph-GA. Results from these studies demonstrated that exposure to 19-Ph-GA at a relatively high concentration (10 μ M) did not significantly increase the rate of oxygen consumption in contrast to exposure to GA which resulted in two-fold increases in the rates of oxygen consumption (Fig. 2D). These results demonstrated that 19BQAs such as 19-Ph-GA were less redox active and had significantly decreased toxicity relative to GA in 5Y cells.

19BQAs are potent inducers of heat shock proteins. Previously, we have demonstrated that 19BQAs inhibit Hsp90 ATPase activity and induce cytotoxicity in human cancer cells (Chang *et al.*, 2014). We therefore performed the experiments to examine the efficacy of 19BQAs as Hsp90 inhibitors in dopaminergic 5Y cells. Screening was carried out at nanomolar concentrations (250-500nM) for 16h and results from these experiments (Fig. 3) clearly show that many 19BQAs including 19-Ph-GA are potent inducers of HSPs. At these low concentrations examined of 19-Ph-GA, significant degradation of prosurvival kinases (p-Akt, Raf-1) did not occur but treatment with higher concentrations (5-10 μ M) of 19-Ph-GA resulted in apparent degradation of p-Akt and Raf-1 (data not shown). These results also demonstrate that many 19-substituted geldanamycin analogs, although not all, are comparable or superior to the clinically tested Hsp90 inhibitor 17-AAG in their ability to induce protective HSPs in 5Y cells. In subsequent studies, we focused on 19-Ph-GA to demonstrate proof of principle.

Overexpression of A53T α -synuclein (α -Syn) resulted in protein handling changes and toxicity. We utilized adenoviral transduction of 5Y cells with mutant A53T α -synuclein (A53T α -Syn) as a cellular model. Interestingly, the overexpression of A53T α -Syn perturbed several key protein handling systems that have been previously shown to be important for determining the fate of misfolded or aggregated proteins (Dimant *et al.*, 2012; Ebrahimi-Fakhari, McLean, *et al.*, 2012; Xiong *et al.*, 2013). As shown in Figure 4, increased expression of A53T, but not WT,

α -Syn led to significant dose dependent growth inhibition of 5Y cells (Fig. 4A) and these data correlated with decreased proteasome activity (Fig. 4B) suggesting that impaired proteasome function may be associated with A53T α -Syn toxicity as previously reported in PC12 cells (Stefanis *et al.*, 2001). In addition to the ubiquitin proteasome system (UPS), the autophagy-lysosome pathway (ALP), molecular chaperones and the ER stress response/unfolded protein response (UPR) all work in concert to maintain intracellular protein homeostasis (Olanow and McNaught, 2011; Xiong *et al.*, 2013; 2014). In these studies, we found that overexpression of A53T α -Syn induced the ER stress response and macroautophagy (hereafter referred as autophagy) in 5Y cells as indicated by the sustained increase in the ER stress marker phosphorylated eIF2 α (p-eIF2 α) and the autophagy marker LC3 II (Fig. 4C). To further examine the effect of A53T α -Syn expression on autophagy we treated 5Y cells (previously transduced with A53T α -Syn for 24h) with chloroquine (CQ) for an additional 48h. CQ is a lysosome inhibitor and suppresses the fusion of autophagosomes with lysosomes and blocks autophagic degradation. The levels of LC3 II further increased after CQ treatment excluding the possibility that A53T α -Syn stimulated autophagic flux was due to the blockade of autophagic degradation (Supplementary Fig. 2A). In addition, we also observed enhanced degradation of the autophagy substrate p62 (Fig. 4C) following transduction with A53T α -Syn. These results demonstrated that autophagy was activated by increased A53T α -Syn expression in 5Y cells. Surprisingly, expression of the protective chaperones Hsp70 and Hsp27 was slightly reduced after elevated expression of A53T α -Syn in 5Y cells (Fig. 4C). It is possible that these changes were induced as secondary responses to A53T α -Syn toxicity. Additionally, we were able to detect increases in the formation of high molecular weight α -Syn oligomers in 5Y cells transduced with mutant A53T but not WT α -Syn (Fig. 4D). These bands were not ubiquitinated forms of A53T α -Syn as

indicated by lack of reactivity with an anti-ubiquitin antibody (data not shown). Since the transduction with WT α -Syn did not result in significant toxicity (Fig. 4A), these data are consistent with previous literature (Conway *et al.*, 2000; Kalia *et al.*, 2013) suggesting a link between A53T α -Syn oligomer formation and toxicity in 5Y cells. It is also noteworthy that treatment with the lysosome inhibitor CQ resulted in a greater level of α -Syn oligomers in 5Y cells indicating that autophagy may be an important protective mechanism for α -Syn oligomer degradation (Supplementary Fig. 2B).

19-Ph-GA alleviated A53T α -Syn induced toxicity. Continuous exposure (72h) of 5Y cells to 19-Ph-GA at a concentration of 2.5 μ M resulted in a small but significant loss of cell viability while treatment with higher concentrations showed increased toxicity. Lower concentrations (0.5-1 μ M) of 19-Ph-GA were nontoxic (Fig. 5A). Importantly, we found that 19-Ph-GA was able to significantly alleviate A53T α -Syn induced toxicity. As shown in Figure 5B, after 48h treatment with non toxic concentrations of 19-Ph-GA (0.5-1 μ M), the viability of A53T α -Syn overexpressing cells was increased over 20% and was not significantly different from untreated cells. We have tested pretreatment and co-treatment with 19-Ph-GA, relative to A53T α -Syn transduction, but neither was as effective as post-treatment in terms of improving cell viability (data not shown). In addition, after 48h post-treatment with either 17-AAG or 17-DMAG at the equivalent concentrations (0.5-1 μ M) significant cell death was observed and there was no beneficial effect on A53T α -Syn toxicity (Supplementary Fig. 3). These results demonstrated that exposure to low concentrations of 19-Ph-GA were beneficial and ameliorated A53T α -Syn induced toxicity in 5Y cells.

19-Ph-GA activated heat shock protein response and autophagy that may contribute to the decreased oligomer formation and toxicity of A53T α -Syn. To further examine potential

mechanisms underlying 19-Ph-GA mediated protective effects we characterized biochemical changes modulated by 19-Ph-GA particularly with respect to protein handling systems. As shown in Figure 6A and B, 24h after transduction with A53T α -Syn, exposure of 5Y cells to 19-Ph-GA for 48h resulted in a significant decrease in higher molecular weight α -Syn (HW α -Syn) oligomers while α -Syn monomers were not significantly perturbed. We also blotted α -Syn oligomers using non-denaturing native-PAGE. Interestingly, in these studies treatment with 19-Ph-GA also led to a decrease in native HW α -Syn oligomers as well as some redistribution of HW α -Syn oligomers to smaller oligomers (~76kD) (Fig. 6C). 19-Ph-GA might mediate its protective effects via induction of Hsp70 and Hsp27 to refold mutant A53T α -Syn and prevent higher molecular weight oligomer formation in addition to stimulation of autophagy causing oligomer degradation. However, by the end of 48h treatment we only observed the concomitant induction of Hsp70 and Hsp27 but not induction of LC3 II (Fig. 6D). To further examine potential alterations of HSP response and autophagic flux induced by 19-Ph-GA we conducted a time-course study and found that 19-Ph-GA was able to induce sustained expression of Hsp70 and Hsp27 in 5Y cells while it stimulated a temporal autophagic flux particularly during the early phase (up to 24h) of drug treatment (Fig. 6E). Since 19-Ph-GA did not modulate the ER stress response as indicated by the expression of p-eIF2 α (Fig. 6D) and had little effect on A53T α -Syn induced proteasomal inhibition (Supplementary Fig. 4), the temporal autophagy activated by 19-Ph-GA might be particularly important as an alternative pathway for α -Syn oligomer degradation. Indeed, we found that 19-Ph-GA not only could significantly restore the expression of Hsp70 and Hsp27 in A53T α -Syn overexpressing cells after 48h post-treatment, but also significantly stimulated an early induction of autophagy at 24h (Fig. 6F and G). Taken together, our results suggest that both the ability of 19-Ph-GA to induce HSP expression as well as to

activate autophagy should be considered as potential mechanisms for decreasing A53T α -Syn oligomer formation and toxicity in 5Y cells.

19-Ph-GA significantly down regulated mTOR/p70S6K signaling in A53T α -Syn overexpressing cells. MTOR (mTORC1) regulates many signaling proteins involved in various cellular processes including cell growth, protein synthesis and autophagy (Maiese *et al.*, 2013). Since mTOR has been reported to be a client protein of Hsp90 (Moulick *et al.*, 2011), we hypothesized that 19-Ph-GA might modulate mTOR activity in 5Y cells overexpressing A53T α -Syn. In contrast to a recent study (Jiang *et al.*, 2013), we observed a sustained increase in the levels of LC3 II after overexpression of either A53T or WT α -Syn in 5Y cells while the concomitant induction of p-mTOR and p-p70S6K was only detected in A53T α -Syn overexpressing cells (Fig. 7). Moreover, post-treatment with 19-Ph-GA for 48h significantly attenuated the levels of p-mTOR and p-p70S6K in A53T α -Syn overexpressing cells but had little effect on the amount of LC3 II (Fig. 8). It is noteworthy that increased A53T α -Syn expression activated mTOR/p70S6K signaling in both a time and dose-dependent manner (Supplementary Fig. 5) suggesting a potential role of mTOR/p70S6K signaling in A53T α -Syn induced toxicity.

Discussion

In this study, we demonstrated that 19-Ph-GA, a novel BQA Hsp90 inhibitor induced significantly less toxicity relative to its parent quinone GA while still retaining the ability to induce HSPs in human dopaminergic 5Y cells. To evaluate the potential therapeutic effects of 19-Ph-GA in PD, we established an A53T α -Syn cell model using transient adenoviral transduction of human mutant A53T α -Syn in 5Y cells. Our results demonstrated that 19-Ph-GA could effectively decrease the formation of high molecular mass α -Syn oligomers and toxicity of A53T α -Syn. 19-Ph-GA triggered a sustainable increase in expression of HSP and the temporal induction of autophagy both of which may play a role in the protective effects observed with 19-Ph-GA. Intriguingly, we found that mTOR/p70S6K signaling was activated by overexpression of A53T but not WT α -Syn and 19-Ph-GA decreased mTOR activation. It is possible that this effect may also contribute to the protective mechanism of 19-Ph-GA against A53T α -Syn induced toxicity but requires further investigation.

The mechanisms of α -Syn degradation have not been fully elucidated, but protein handling systems have been shown to play a role in modulating the levels of intracellular α -Syn via multiple pathways: the 20/26S proteasome and/or autophagy mediates degradation of α -Syn to alleviate intracellular protein load (Sarkar *et al.*, 2007; Ebrahimi-Fakhari *et al.*, 2011); and α -Syn could be chaperoned by heat shock proteins including Hsp70 and Hsp27 to limit the formation of toxic oligomers (Auluck *et al.*, 2002; Zourlidou *et al.*, 2004; Kilpatrick *et al.*, 2013). Interestingly, overexpression of α -Syn particularly its pathogenic form A53T α -Syn has been shown to impair functions of the UPS or CMA and lead to further accumulation of α -Syn (Ebrahimi-Fakhari, Wahlster, *et al.*, 2012). In this study, we found that increased levels of A53T α -Syn resulted in a significant loss of cell viability and that was associated with the formation of

α -Syn oligomers as well as alterations in protein handling systems including decreased protein levels of Hsp27, Hsp70 and decreased proteasomal activity. Overexpression of WT α -Syn did not induce oligomer formation and toxicity in 5Y cells as observed with A53T α -Syn overexpression suggesting that formation of oligomers was important for triggering the toxicity of α -Syn.

Inhibiting Hsp90 leads to compensatory induction of HSPs and has been proposed as an effective strategy for the therapy of neurodegenerative diseases (Luo *et al.*, 2010; Blair *et al.*, 2014). In fact, because of their ability to induce Hsp70 several Hsp90 inhibitors have recently been tested in a rat α -Syn PD model (Putcha *et al.*, 2010; McFarland *et al.*, 2014). However, the failure of these candidate molecules in ameliorating dopaminergic neurodegeneration indicated that the compensatory upregulation of Hsp70 by itself may be insufficient to protect against α -Syn toxicity (Zourlidou *et al.*, 2004; Shimshek *et al.*, 2010). The BQA class of Hsp90 inhibitors including GA and 17-AAG has been found to be effective in inducing both multiple HSPs and autophagy (Finn *et al.*, 2005; Riedel *et al.*, 2010; Watanabe *et al.*, 2010). However, the protective effects of BQAs are accompanied by off-target toxicities limiting their use in neurodegenerative diseases. Therefore, we examined the ability of novel 19-substituted BQA analogs to alleviate toxicity induced by α -Syn overexpression. 19-substituted BQA analogs were synthesized to prevent direct conjugation with thiols at the 19 position (Kitson *et al.*, 2013) and studies have demonstrated that 19 substitutions resulted in less toxicity when compared to their unsubstituted parent BQAs (Chang *et al.*, 2014). Our data demonstrated that 19-Ph-GA was able to induce HSP expression and autophagy and exerted significant protective effects when tested in an A53T α -Syn cell model. After treatment with 19-Ph-GA we observed a significant reduction in HW α -Syn oligomers as well as a redistribution of HW α -Syn oligomers to smaller oligomers. These

effects on HW α -Syn oligomers following treatment with 19-Ph-GA may be the result of induction of Hsp70, Hsp27 and/or autophagy.

MTOR (TORC1) plays a key role in many cellular processes including cell growth, proliferation, metabolism and protein synthesis (Laplante and Sabatini, 2012). Its activity can be monitored by following the phosphorylation of substrates such as p70S6 kinase (p70S6K) at Thr389 (Yip *et al.*, 2010). The inhibition of mTOR typically results in induction of autophagy and has been proposed as an attractive strategy for the therapy of misfolded protein diseases (Bové *et al.*, 2011). However, in some cases mTOR inhibition does not necessarily result in autophagy but instead autophagy may be positively regulated by mTOR (Zeng and Kinsella, 2008) or may even be mTOR-independent (Sarkar, 2013). Our results demonstrated that overexpression of either A53T or WT α -Syn in 5Y cells could induce autophagy while the activation of mTOR was only detected in A53T α -Syn overexpressing cells. In addition, post-treatment with 19-Ph-GA for 48h significantly blocked the activation of mTOR/p70S6K signaling whereas it had no significant effect on LC3 II levels in A53T α -Syn overexpressing cells. These results suggest that mTOR activation is not necessary for synuclein induced autophagy and a number of previous studies have demonstrated overexpression of pathogenic forms of α -Syn could activate compensatory autophagy in response to either impaired chaperone-mediated autophagy (CMA) or UPS activity (Stefanis *et al.*, 2001; Cuervo *et al.*, 2004; Xilouri *et al.*, 2009).

The activation of mTOR/p70S6K signaling may be important for A53T α -Syn induced toxicity in 5Y cells although the mechanisms remain unclear. It is possible that mTOR activated downstream p70S6K to upregulate protein synthesis including the synthesis of aberrant proteins such as A53T α -Syn and resulted in proteolytic stress and toxicity as previously reported in a cell

model of Huntington's disease (King *et al.*, 2008). Recently, mTOR has also been found to drive the phosphorylation status of transcriptional factor EB (TFEB) preventing it from translocating to the nucleus and activating lysosome biogenesis (Decressac *et al.*, 2013). Since lysosomes are critical to the process of autophagy, overexpression of A53T α -Syn might activate mTOR to inhibit lysosome biogenesis and lead to incomplete autophagic degradation of α -Syn oligomers and toxicity in 5Y cells. Our data also demonstrated that 19-Ph-GA was very effective in decreasing the levels of p-mTOR and p-p70S6K in A53T α -Syn overexpressing cells. Under stress or disease conditions mTOR/p70S6K signaling may be over activated and since mTOR has been reported as an Hsp90 client protein (Moulick *et al.*, 2011), Hsp90 inhibitors like 19-Ph-GA may be particularly effective.

In summary, we demonstrated that 19-Ph-GA an analog of GA showed decreased toxicity when compared to GA while maintaining the ability to induce a robust HSP response as well as modulating the activation of autophagy in 5Y cells. The multiple protective effects of 19-Ph-GA rendered 5Y cells more resistant to mutant A53T α -Syn induced adverse effects (Fig. 9). The data provide proof of principle that compounds which stimulate the HSP response and autophagy such as 19-Ph-GA may be worthy of further translational studies for the therapy of protein folding diseases including PD.

Authorship Contributions

Participated in research design: Xiong, Siegel, and Ross

Conducted experiments: Xiong

Contributed new reagents or analytic tools: Zhou, Freed, Kitson, and Moody

Performed data analysis: Xiong, Siegel, and Ross

Wrote or contributed to the writing of the manuscript: Xiong, Siegel and Ross

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Footnotes

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DS, RK, CJM, DR and the University of Colorado have a patent interest in 19-substituted benzoquinone ansamycins.

To whom correspondence should be addressed: David Ross, Department of Pharmaceutical Sciences, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of Colorado Anschutz Medical Campus, 12850 E. Montview Blvd, Aurora, CO 80045. Tel.: 303-724-7265; Fax: 303-724-7266; E-mail: David.Ross@ucdenver.edu.

Figure Legends

Figure 1. Chemical structures of benzoquinone ansamycins (BQA). Substitutions at the 19-position (R) include phenyl (Ph), 4-phenyl-phenyl (Ph-Ph), naphthyl, 4-methoxy-phenyl (Ph-OMe), 4-morpholinyl-phenyl (Ph-Mor), hydroxymethyl (HM), dimethylamino (DMA), methyl (Me).

Figure 2. 19BQAs exerted decreased toxicity compared with their parent quinone GA. (A) Growth inhibition induced by 19BQAs in 5Y cells. Growth inhibition was measured using the MTT assay. Cells were treated with BQAs for 4hr then allowed to grow for an additional 72hr. (B) Cells were treated with GA and 19-Ph-GA at 0.25 μ M for 24h and harvested for determination of cell number by using a trypan blue exclusion assay. (C) Apoptosis was measured using annexin V/PI cell staining in combination with flow cytometry in cells after exposure to the equivalent concentration (5 μ M) of GA or 19-Ph-GA for 16h. (D) Oxygen consumption was measured in cells in the absence (basal) or presence of the indicated compounds. Measurements were made over 10min using a Clark electrode at 37°C. Menadione was included as a redox-cycling quinone (positive control). Results are expressed as the mean \pm SD, n=3. *p<0.05, ***p<0.001 is considered significantly different from basal by one-way ANOVA using Dunnet's multiple comparison test.

Figure 3. Treatment with 19BQAs induced HSPs in 5Y cells. Immunoblot analysis of biomarkers of Hsp90 inhibition following treatment with BQAs at the indicated concentrations for 16h. Note that many 19BQAs induced potent expression of Hsp70 and Hsp27.

Figure 4. Overexpression of human mutant A53T α -Syn induced protein handling changes and toxicity in 5Y cells. (A) One day after seeding, cells were transduced with mutant A53T α -Syn (MOI from 100 to 800 pfu/cell) and WT α -Syn (MOI 400 pfu/cell) for 72h. Cell viability was then determined by the MTT assay. (B) Overexpression of A53T α -Syn inhibited intracellular 20/26S proteasomal activity (chymotrypsin-like active site) in a dose-dependent manner. (C) Hsp70 and Hsp27 protein levels and induction of the ER stress response and autophagy following transduction with A53T α -Syn. (D) MOI-dependent increase in higher molecular weight α -Syn oligomers was detected 72h after transduction with A53T α -Syn but not WT α -Syn and GFP adenovirus vector control (MOI 400 pfu/cell). β -actin was included as a loading control. Values in (A) and (B) are presented as mean \pm SD, (n=3); *p<0.05 is considered significant to control group (one-way ANOVA using Dunnet's multiple comparison test).

Figure 5. 19-Ph-GA significantly attenuated A53T α -Syn induced toxicity in 5Y cells. (A) Treatment with low concentrations of 19-Ph-GA (0.5-1 μ M) for 72h was not cytotoxic to 5Y cells. (B) After 24h incubation with adenovirus expressing mutant A53T α -Syn (400 pfu/cell), cells were exposed to the indicated concentrations of 19-Ph-GA for 48h after which cell viability was determined by the MTT assay. Triplicate treatments in 48-well plates were used. Results represent one experiment typical of n=5. Values are presented as mean \pm SD, (n=3); *p<0.05, **p<0.01 is considered significant compared with control in (A) and relative to A53T α -Syn group in (B), respectively, by one-way ANOVA using a Tukey's multiple comparison test.

Figure 6. 19-Ph-GA significantly reduced A53T α -Syn oligomer formation through induction of heat shock protein responses and autophagy. (A, B) α -Syn oligomers

(denatured) were significantly reduced after 48h post-treatment of 5Y cells with 19-Ph-GA. (B) Fold changes of α -Syn oligomers in (A) after the drug treatment are normalized to respective controls and estimated by densitometry. (C) Exposure of 5Y cells to 19-Ph-GA resulted in decreased levels of native high molecular weight α -Syn oligomers as well as an increase in the smaller (76 kDa) oligomer species. (D) After 48h post-treatment with 19-Ph-GA, Hsp70 and Hsp27 protein levels were elevated 2- to 3-fold but biomarkers of autophagy and ER stress were not significantly perturbed. (E) Time-course study showed that 19-Ph-GA was able to upregulate both HSPs and LC3 II expression in 5Y cells. Note that exposure to 19-Ph-GA for 24h induced the maximum increase in LC3 II suggesting 19-Ph-GA stimulated a temporal autophagic flux in 5Y cells. (F, G) Significant induction of HSPs and LC3 II could be detected in A53T α -Syn overexpressing cells after post-treatment with 19-Ph-GA (0.5 μ M) for 24h suggesting that HSP response and autophagic flux were stimulated by 19-Ph-GA at relatively early time points. β -actin was included as a loading control. Representative blot from three repeated experiments was showed for each figure. (G) Fold changes of Hsp70, Hsp27 and LC3 II in (F) after the drug treatment at the indicated times are normalized to respective controls and estimated by densitometry. Values in (B) and (G) are presented as mean \pm SD, (n=3); *p<0.05 considered significant compared with A53T α -Syn group by one-way ANOVA using a Tukey's multiple comparison test.

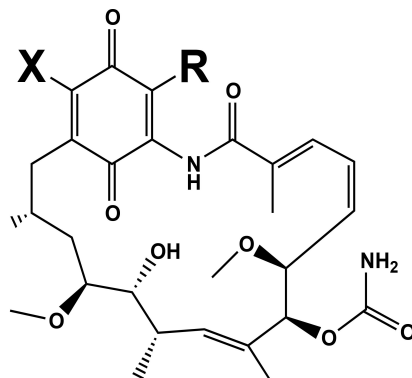
Figure 7. MTOR/p70S6K signaling was activated by overexpression of A53T but not WT α -Syn. (A) Overexpression of A53T but not WT α -Syn significantly increased the levels of phosphorylated mTOR and p70S6K in 5Y cells. Increased amount of LC3 II was observed in both A53T and WT α -Syn overexpressing cells suggesting that autophagy was not directly

mediated via the mTOR/p70S6K signaling. (B) Fold changes of LC3 II, p-mTOR and p-p70S6K levels in (A) are normalized to respective controls and estimated by densitometry. These values are presented as mean \pm SD, (n=3); *p<0.05 is considered significant relative to control group using Dunnet's multiple comparison test. (C) Increased expression of p-mTOR was observed in A53T but not WT α -syn overexpressing cells. After transduction with adenovirus expressing A53T α -Syn or WT α -Syn at MOI 400 pfu/cell for 72h, 5Y cells were fixed and immunostained for α -Syn (red), p-mTOR (green) and nuclei (DAPI, blue) and then examined by confocal microscopy. Note that the colocalization of p-mTOR and α -Syn (white arrow) suggesting an interaction between p-mTOR and A53T α -Syn may exist.

Figure 8. 19-Ph-GA decreased mTOR activation that was associated with A53T α -Syn induced toxicity. Post-treatment with 19-Ph-GA for 48h significantly blocked the activation of p-mTOR and p-p70S6K induced by A53T α -Syn. (B) Fold changes of LC3 II, p-mTOR and p-p70S6K levels in (A) are normalized to respective controls and estimated by densitometry. These values are presented as mean \pm SD, (n=3); *p<0.05 is considered significant relative to control group; #p<0.05 is considered significant compared with A53T α -Syn group by one-way ANOVA using a Tukey's multiple comparison test.

Figure 9. Protective effects of 19-Ph-GA on A53T α -Syn induced protein handling changes and toxicity in 5Y cells. Overexpression of A53T α -Syn (labeled in red) resulted in α -Syn oligomer formation and toxicity via perturbing major protein handling systems including increased proteasomal inhibition, ER stress response and decreased levels of HSPs. Autophagy could be activated by overexpression of either A53T or WT α -Syn (labeled in black) excluding

the possibility that autophagic cell death was induced specifically by A53T α -Syn overexpression. MTOR/p70S6K signaling was not directly involved in A53T α -Syn induced autophagy but was positively related to the toxicity of A53T α -Syn. 19-Ph-GA (labeled in blue) activated Hsp70, Hsp27 and autophagy and ameliorated A53T α -Syn induced mTOR activation. Collectively, these mechanisms may contribute to protection against A53T α -Syn oligomer formation and toxicity.



| BQA | X | R |
|----------------|--|-------------------------------|
| GA | OCH ₃ | H |
| 17-AAG | NHCH ₂ CHCH ₃ | H |
| 17-DMAG | NHCH ₂ CH ₂ N(CH ₃) ₂ | H |
| 19-Ph-GA | OCH ₃ | phenyl (Ph) |
| 19-Ph-Ph-GA | OCH ₃ | 4-phenyl-phenyl (Ph-Ph) |
| 19-Naphthyl-GA | OCH ₃ | naphthyl |
| 19-Ph-OMe-GA | OCH ₃ | 4-methoxy-phenyl (Ph-OMe) |
| 19-Ph-Mor | OCH ₃ | 4-morpholinyl-phenyl (Ph-Mor) |
| 19-HM-GA | OCH ₃ | hydroxymethyl (HM) |
| 19-DMA-GA | OCH ₃ | dimethylamino (DMA) |
| 19-Me-GA | OCH ₃ | methyl (Me) |

FIGURE 1.

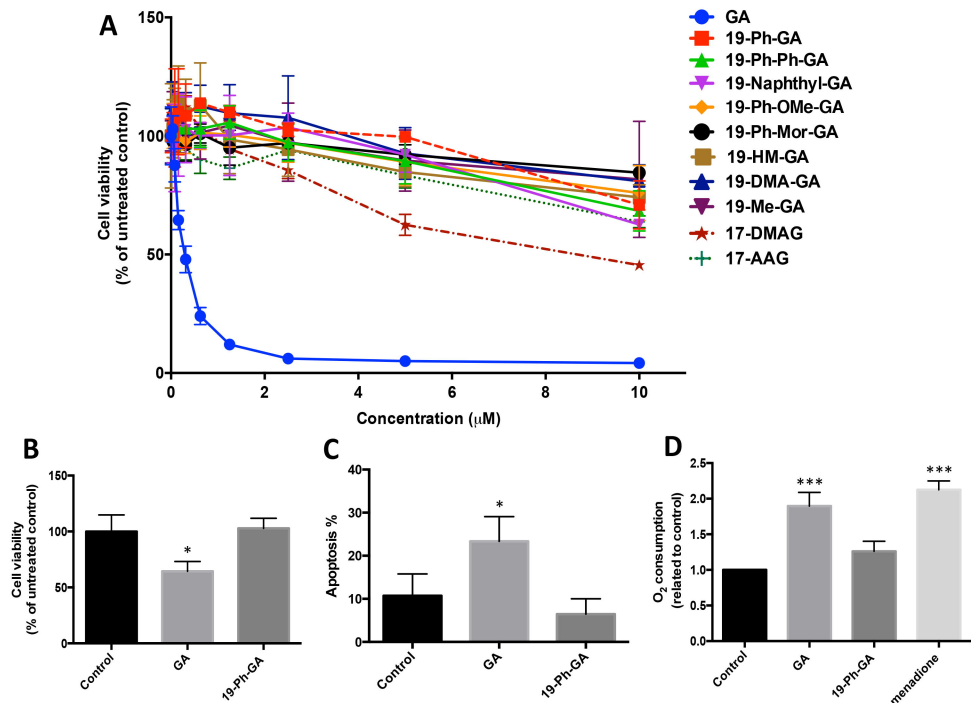


FIGURE 2.

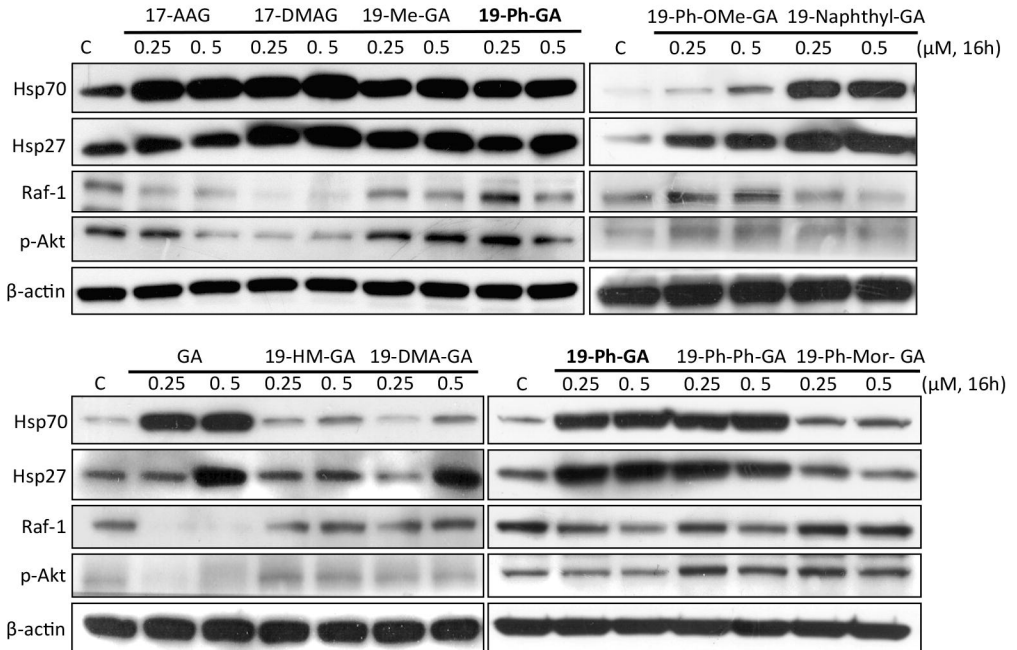


FIGURE 3.

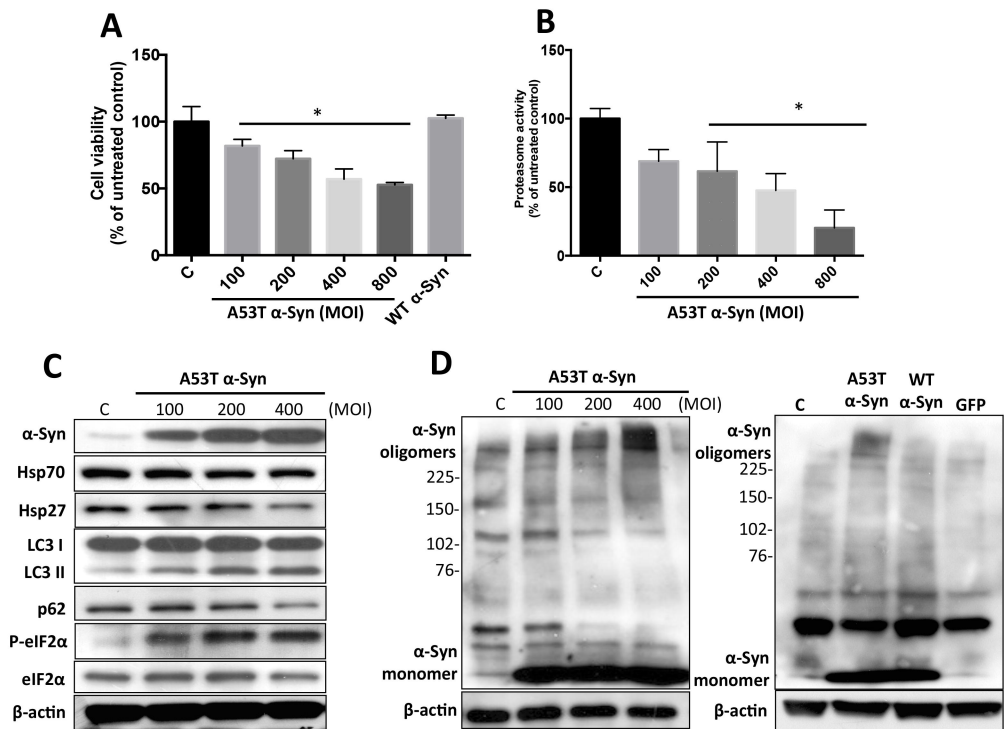


FIGURE 4.

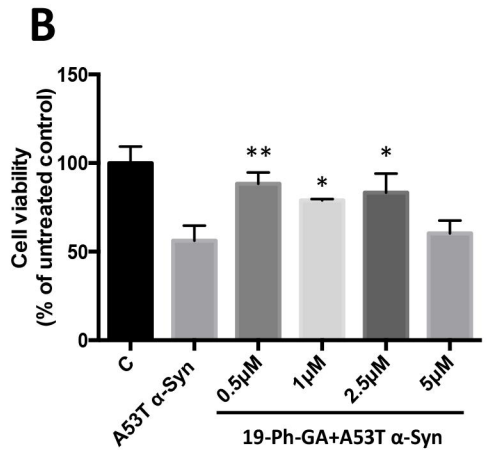
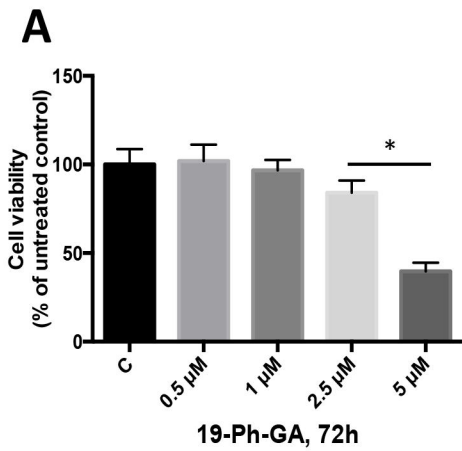


FIGURE 5.

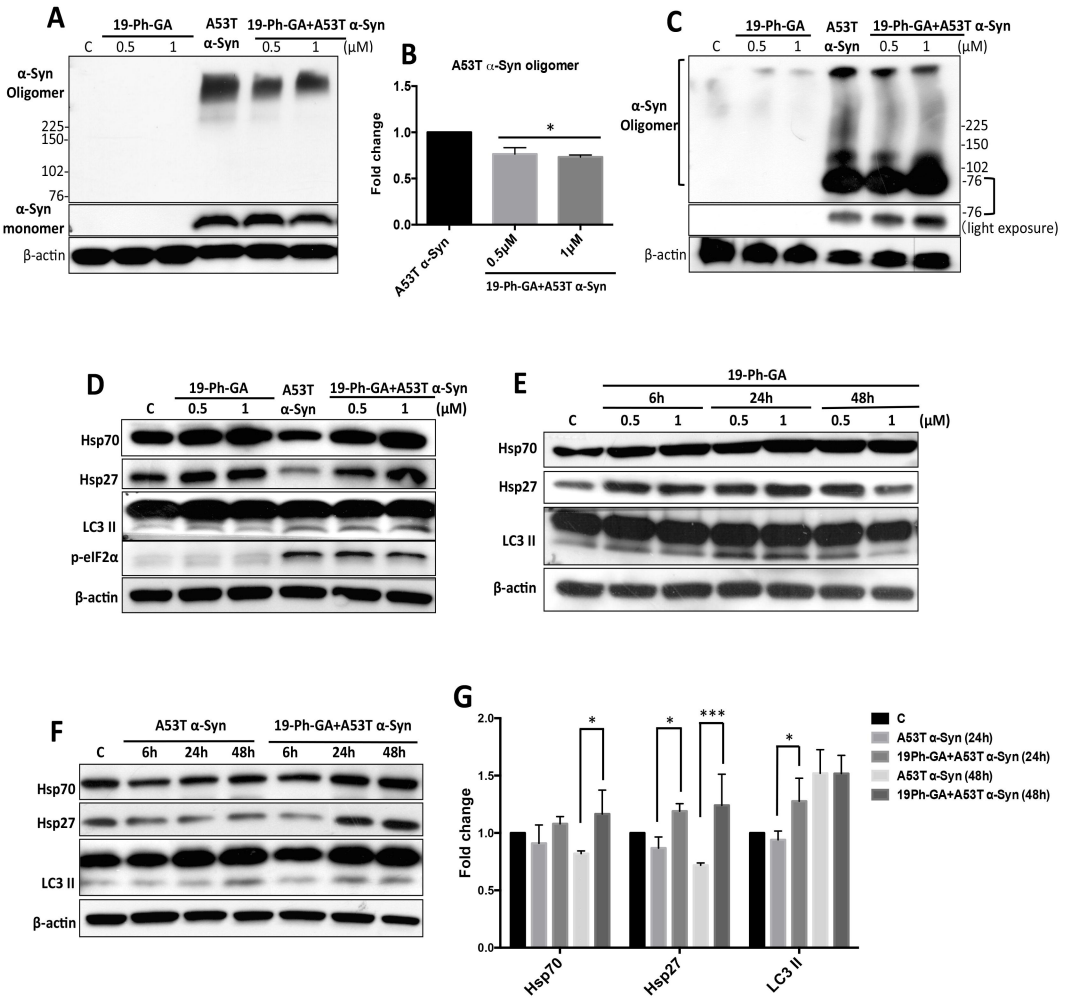


FIGURE 6.

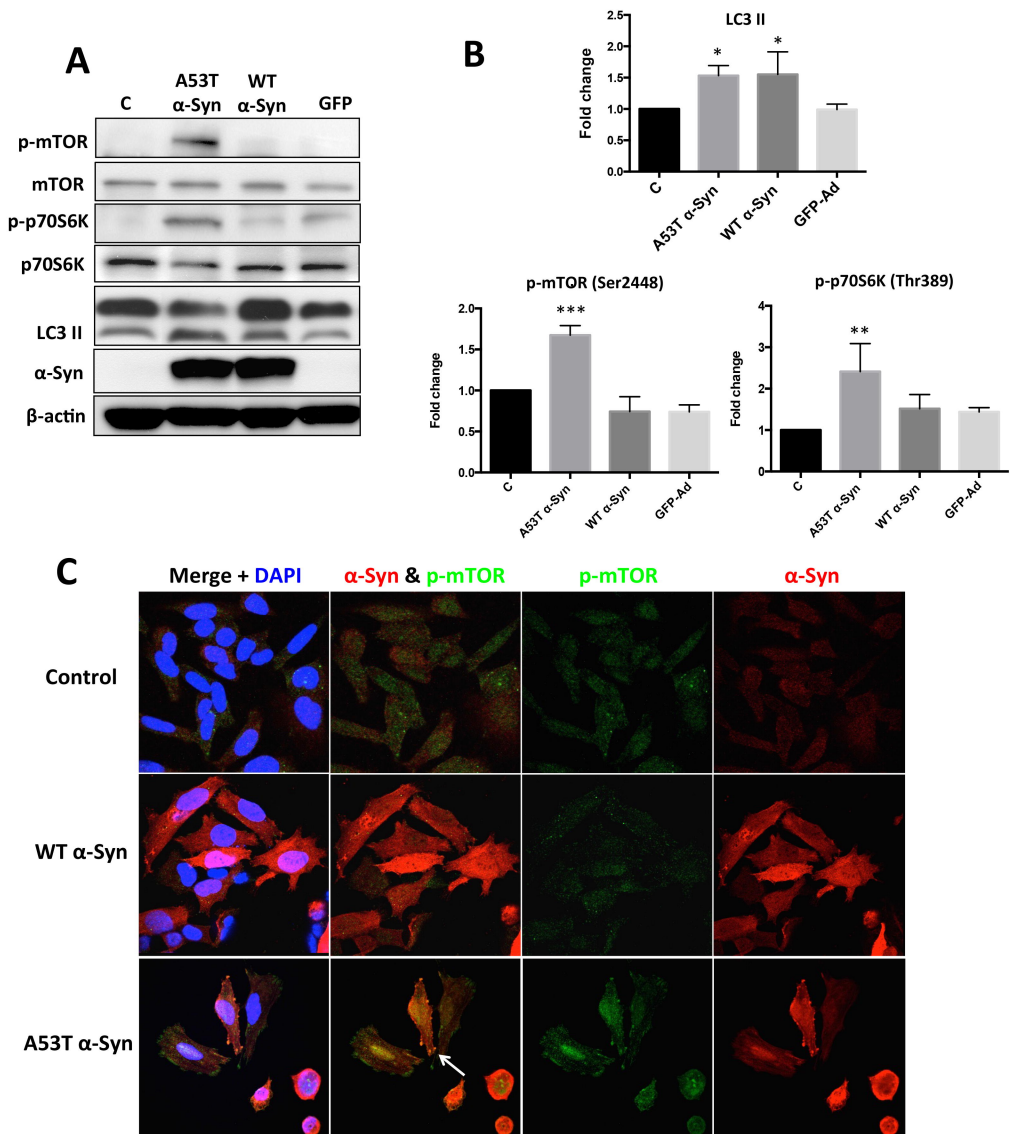


FIGURE 7.

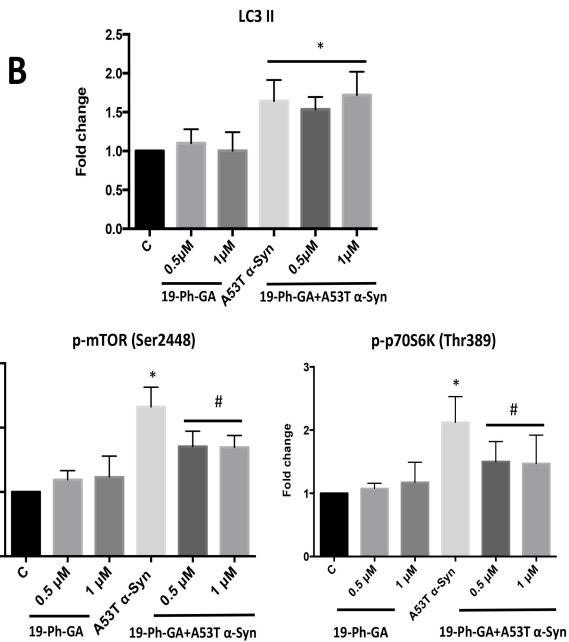
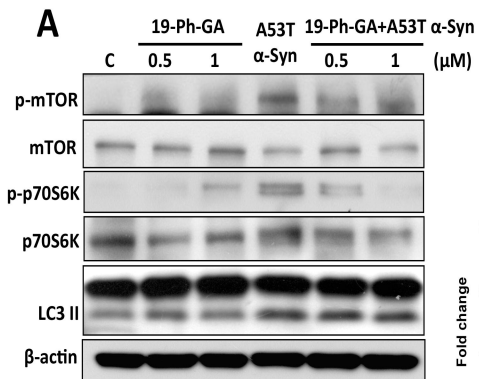


FIGURE 8.

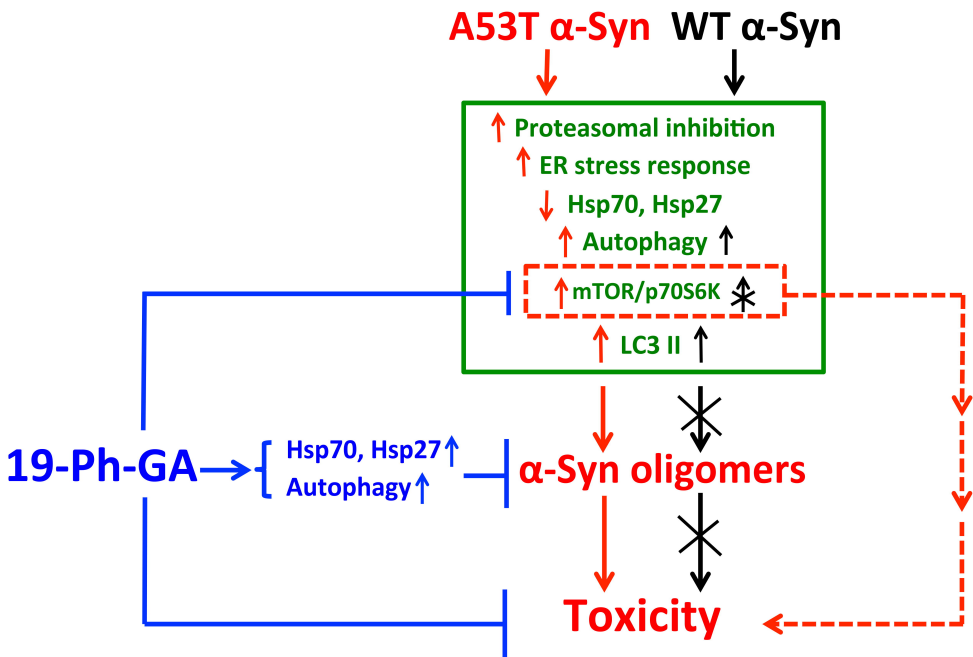


FIGURE 9.