MOL Manuscript #101451

SUPPORTING INFORMATION

for

MOLECULAR PHARMACOLOGY

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.*)

(Methods for supplementary data including immunoblot analysis, MTT growth inhibition assay, proteasomal activity assay and immunocytochemistry are described in Materials and Methods)

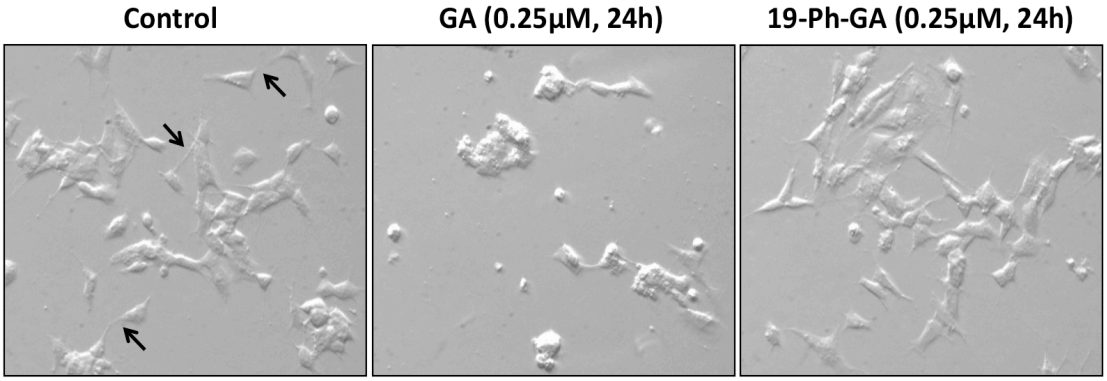

Figure 1. Morphological changes of 5Y cells following treatment with GA and 19-Ph-GA. Bright field microscope images showed that treatment of 5Y cells with GA at 0.25 μM for 24h resulted in lost of cell neurites (black arrows) while exposure to 19-Ph-GA at the equivalent dose did not affect the morphology of cells.

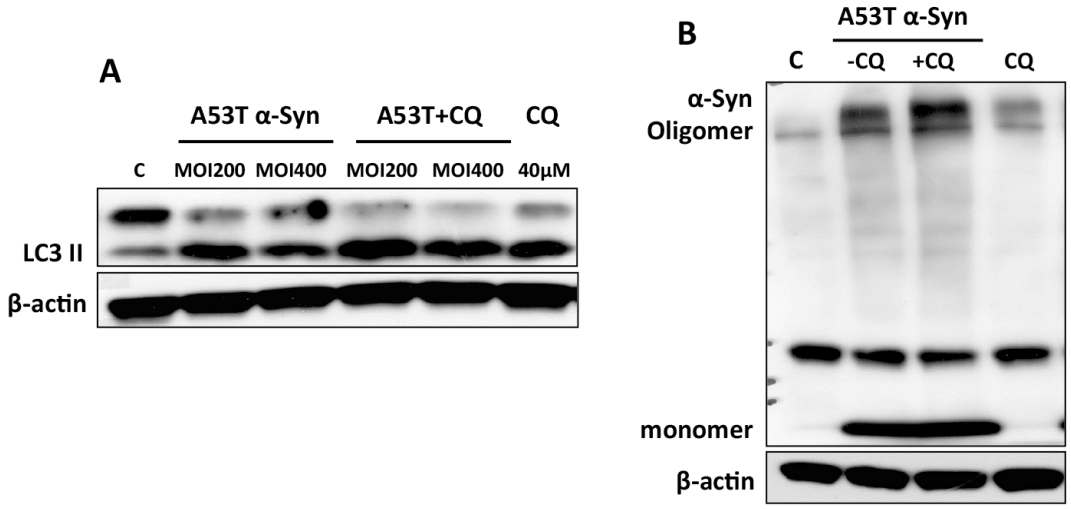

Figure 2. **Autophagy induction in A53T α-Syn overexpressing cells was confirmed by co-treatment with chloroquine (CQ).** (A) 5Y cells were transduced with adenovirus expressing mutant A53T α-Syn at an MOI 200 or 400 pfu/cell for 24h following by incubation with or without CQ for 48h and then processed for immunoblot analysis. Note that CQ is a lysosome inhibitor and suppresses the fusion of autophagosomes with lysosomes and blocks the autophagic degradation. Further increases in amount of LC3 II in the presence of CQ excluded the possibility that A53T α-syn stimulated autophagic flux was due to the blockade of autophagic degradation in 5Y cells. (B) The combined treatment of A53T α-Syn (MOI 400 pfu/cell) with CQ (40 μM) resulted in greater α-Syn oligomers accumulation suggesting autophagy was important for α-syn oligomers degradation in 5Y cells.

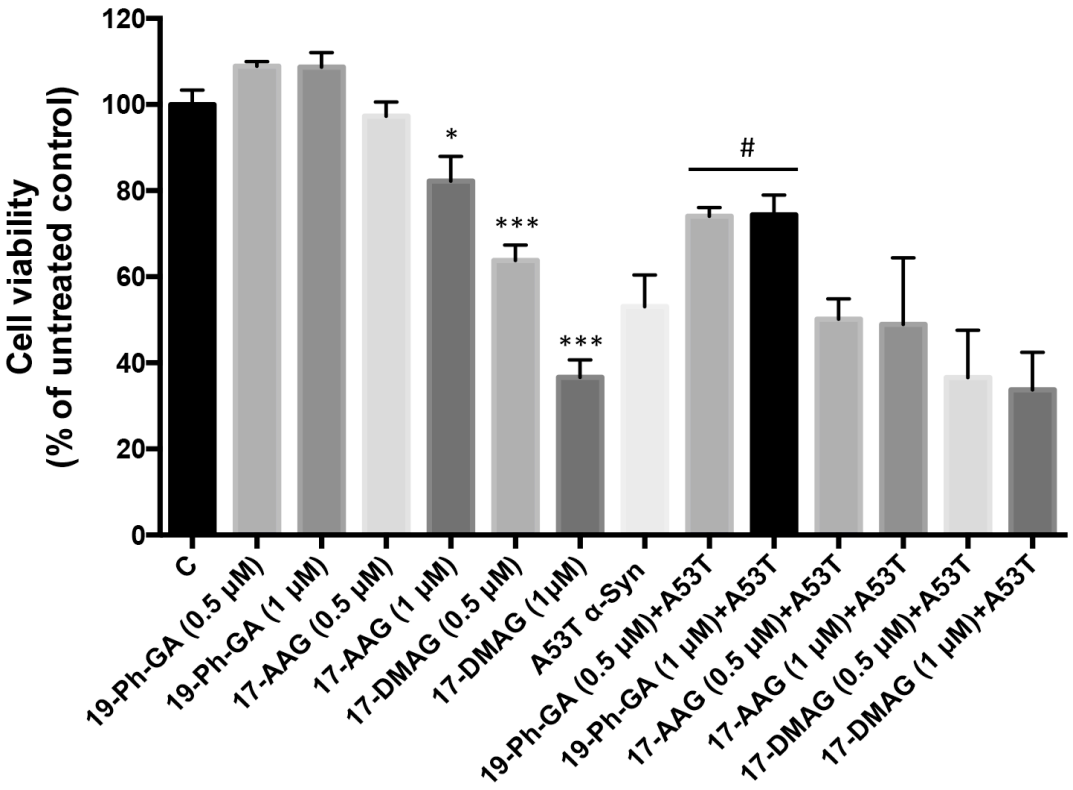

Figure 3. **19-Ph-GA but not 17-AAG and 17-DMAG significantly attenuated A53T α-Syn induced toxicity in 5Y cells.** After 24h incubation with adenovirus expressing mutant A53T α-Syn (400 pfu/cell), cells were exposed to the indicated doses of 19-Ph-GA, 17-AAG or 17-DMAG for 48h after which cell viability was determined by the MTT assay. Triplicate treatments in 48-well plates were used. Values are presented as mean ± SD, (n=3); *$p<0.05$, ***$p<0.001$ 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.

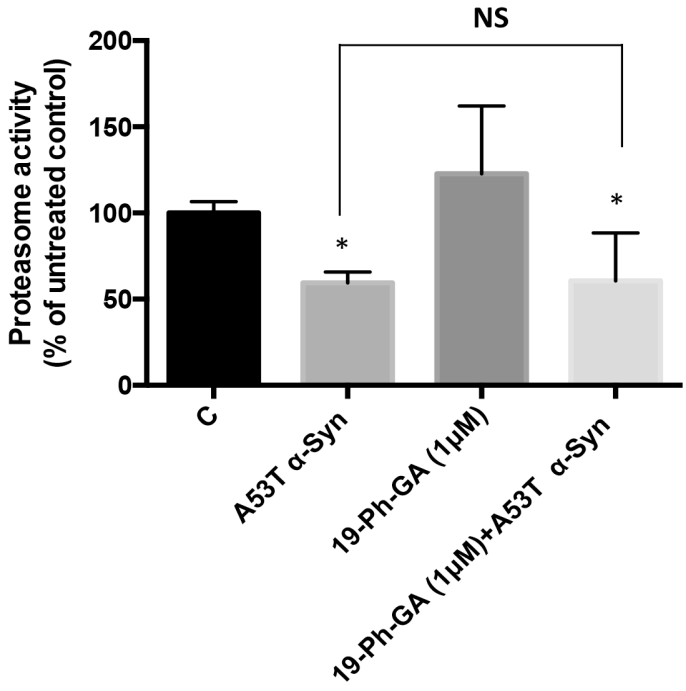

Figure 4. **Post treatment with 19-Ph-GA had little effect on A53T α-syn induced proteasome inhibition.** 5Y cells were transduced with adenovirus expressing mutant A53T α-Syn at an MOI 400 pfu/cell then treated with 19-Ph-GA (1 μM). After 48h cells were harvested and proteasome activities were determined by measuring cleavage of the fluorescent peptide Suc-Leu-Leu-Val-Tyr-AMC (chymotrypsin-like activity) at 380/460 nm. These value are presented as mean ± SD, (n=4); *$p < 0.05$ is considered significant compared with control by ANOVA using Tukey's multiple comparison test.

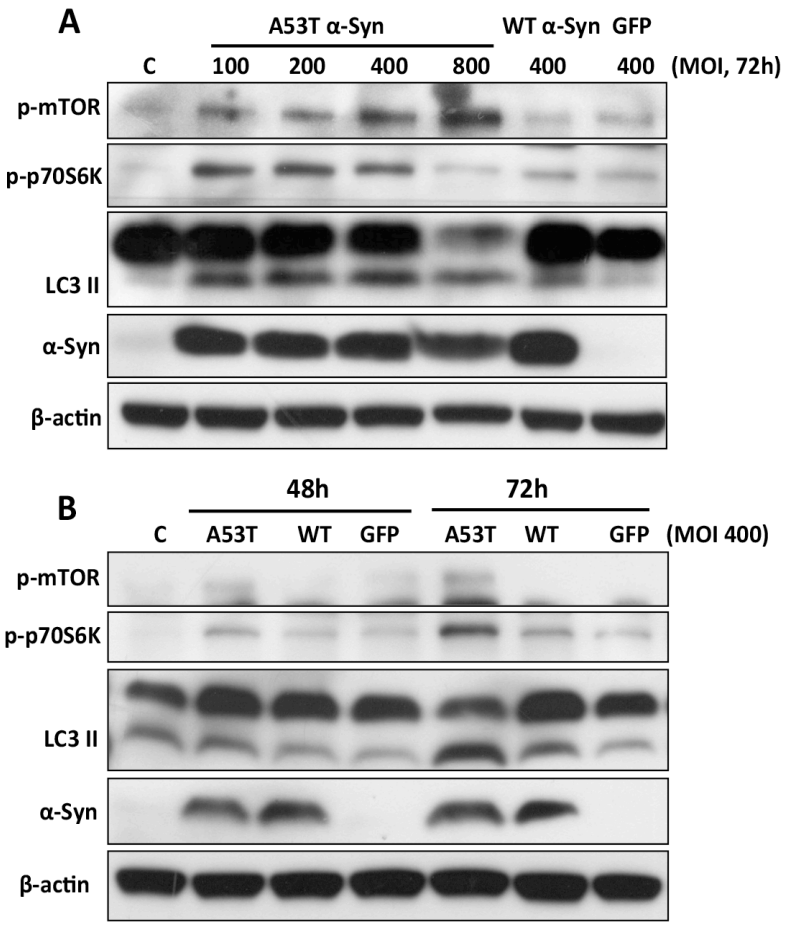

Figure 5. Dose response and time course for the activation of mTOR/p70S6K signaling in A53T α-Syn overexpressing cells. (A) Overexpression of A53T α-Syn upregulated the expression of p-mTOR in a dose-dependent manner in 5Y cells. A sustained increase of p-p70S6K could be detected until transduction with A53T α-Syn at MOI 800 pfu/cell. Note that overexpression of either WT α-Syn or GFP adenoviral vector control did not have any significant effect on the protein levels of p-mTOR and p-p70S6K. (B) Time-course study showed that transduction with A53T α-Syn at MOI 400 pfu/cell increased the expression of p-mTOR and p-p70S6K in a time-dependent manner in 5Y cells.