SUPPLEMENTAL MATERIALS: Pivtoraiko *et. al.*

METHODS

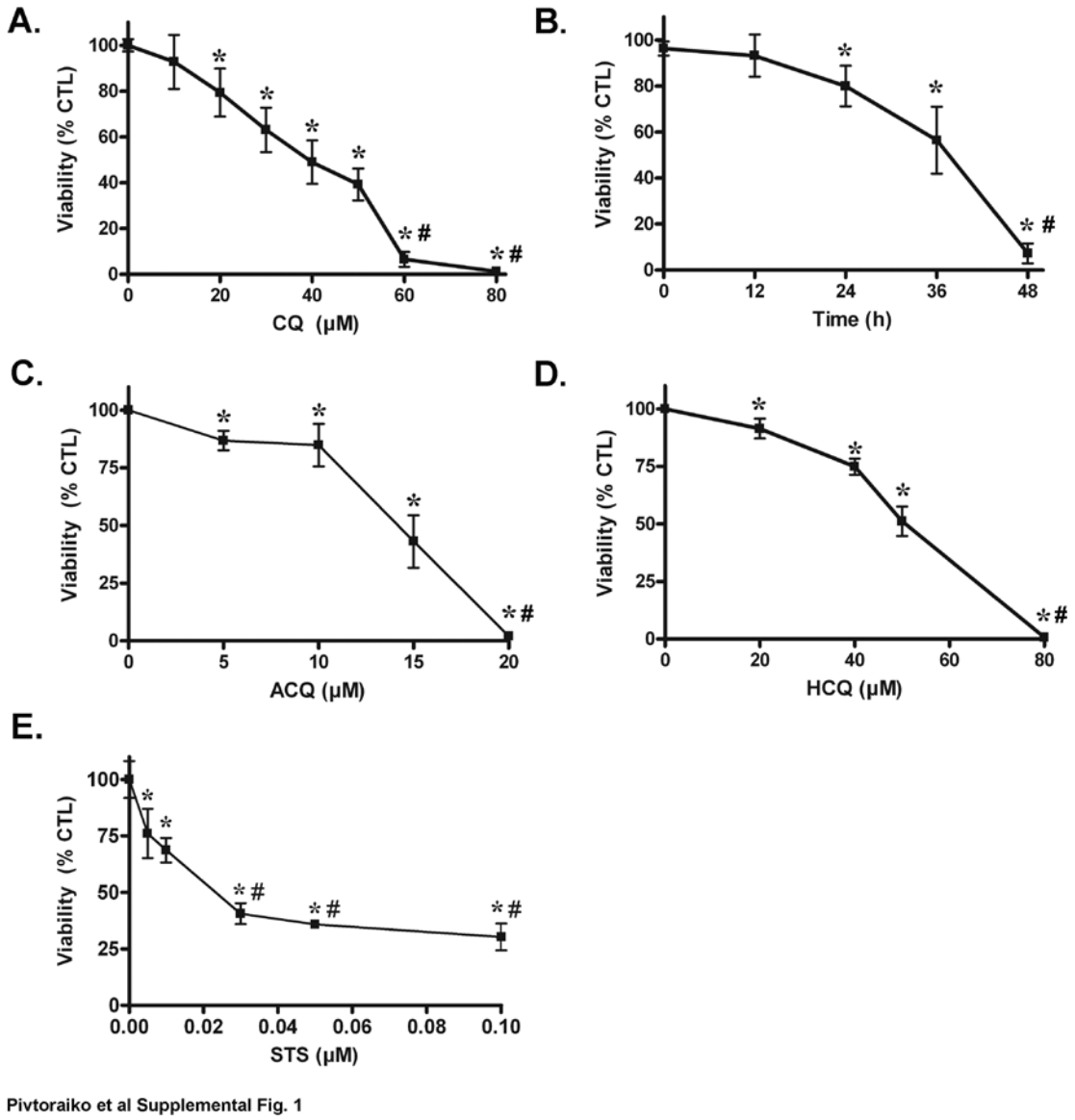
Western Blot.

Whole cell lysates were obtained by detaching cells with Accutase (Innovative Cell Technologies, San Diego, CA) for 5 minutes at 37ºC, followed by neutralization with media containing 10% FBS. Cells and conditioned media were centrifuged (700xg, 5 minutes, 4ºC). The pellet was re-suspended in 1ml of ice-cold PBS and following subsequent centrifugation (700xg, 5 minutes, 4ºC) the pellet was lysed with buffer containing 25mM HEPES, 5mM EDTA, 5mM $MgCl_2$, 1% SDS, 1% Triton X-100, 1mM phenylmethylsulfonyl fluoride, 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktails I and II (Sigma, St. Louis, MO). Cell lysates were sonicated and then centrifuged (10,000xg, 10 minutes, 4ºC), and the resultant supernatants were transferred to fresh tubes.

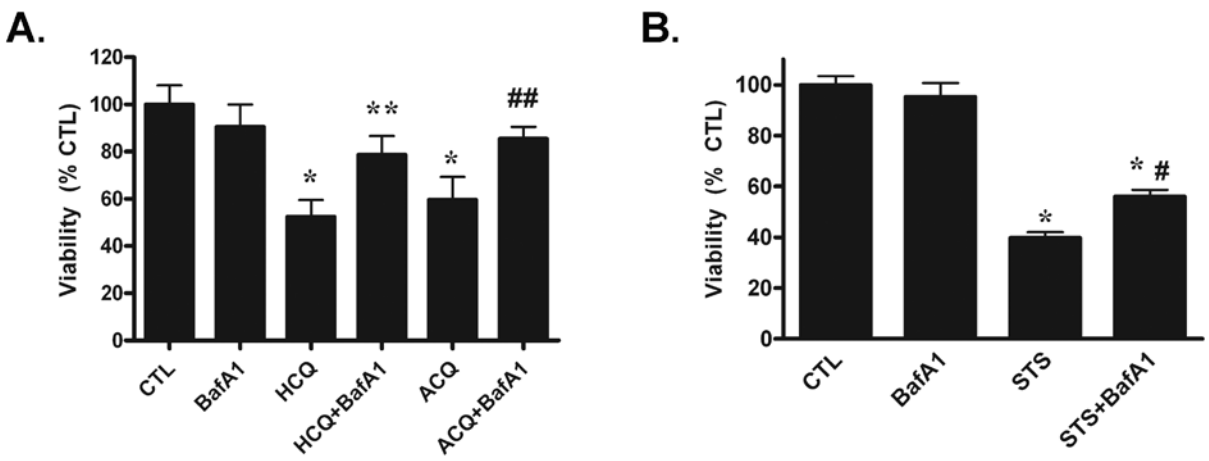
To generate Triton X-100-soluble and -insoluble lysates, the cell pellet following the last spin was re-suspended in buffer containing 50 mM TrisHCl, 175 mM NaCl, 5 mM EDTA and 1% protease inhibitor cocktail (Sigma), sonicated and incubated with 1% Triton X-100 for 30 minutes. The lysates were then centrifuged (15,000xg, 1 hour, 4ºC), and the supernatants comprising the Triton X-100 soluble fraction were transferred to fresh tubes. Triton X-100 insoluble fractions were generated by re-suspending and sonicating the pellets in the same buffer containing 2% SDS.

Protein concentrations were determined using BCA assay (Thermo Scientific, Rockford, IL). Equal amounts of protein were electrophoresed on SDS-polyacrylamide gel and transferred to PVDF membranes (BioRad). To detect microtubule associated protein light chain-3 (LC3), a selective marker of AV accumulation (Klionsky *et al.* 2007) and α-synuclein, membranes were blocked in 5% milk in 1X wash buffer (1X TBS containing 0.1% Tween 20) for 1h at room temperature and subsequently incubated with either rabbit anti-LC3 (Abgent, San Diego, CA) or mouse anti-α-synuclein (BD Biosciences, San Jose, CA) in 5% milk overnight at 4ºC. Blots were then washed and incubated with secondary antibody (LC3: goat anti-rabbit IgG, Bio-Rad, Hercules, CA; α-synuclein: horse anti-moue IgG, Cell Signaling, Danvers, MA) in blocking buffer for 1h at room temperature and subsequently washed. CD was detected using a goat polyclonal primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and donkey anti-goat secondary antibody (Jackson Immunoresearch, West Grove, PA) in blocking buffer consisting of 5% goat serum in 1X wash buffer. Signal was detected using Amersham™ ECL™ Chemiluminescence (GE Healthcare, Piscataway, NJ).

After detection of LC3, α-synuclein and CD, blots were stripped using Restore Western Blot stripping buffer (Thermo Scientific) and probed for protein loading using either mouse anti-actin (Sigma) for LC3 and α-synuclein, or β-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA) for CD. Blots were scanned for densitometric analysis using UN-SCAN-IT gel™ gel analysis software (Silk Scientific, Inc., Orem, UT).

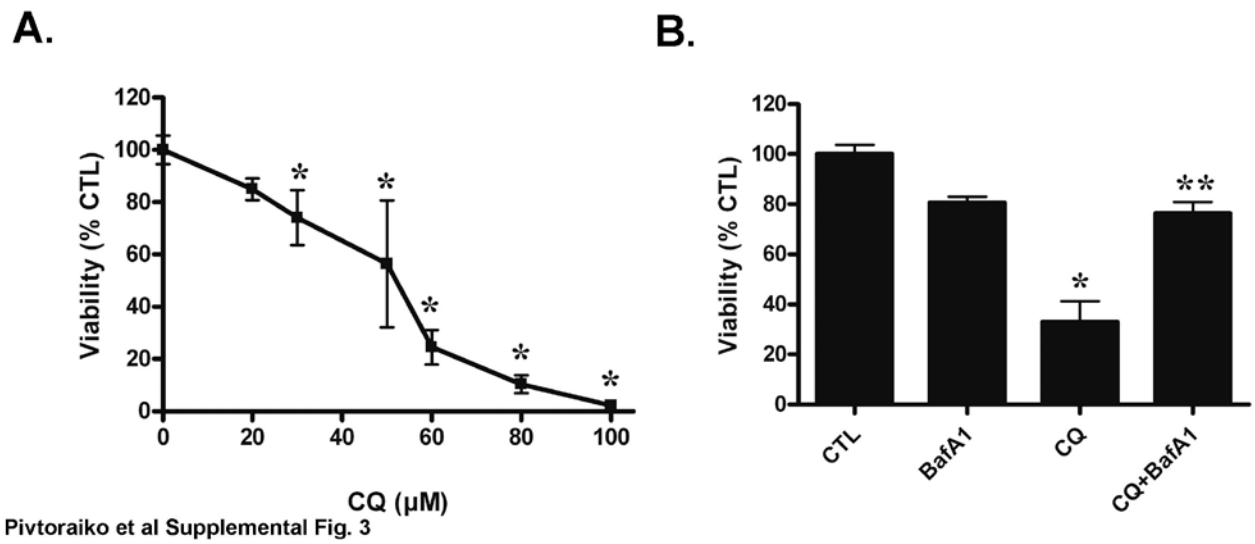


Supplemental Fig. 1. Stimulus-induced death of SH-SY5Y cells. Naïve SH-SY5Y cells were treated with chloroquine (CQ, A, B), amodiaquine (ACQ, C), hydroxychloroquine (HCQ, D) or staurosporine (STS, E) for 48h. Cell viability was assessed using fluorogenic calcein conversion assay. All death stimuli induced significant and concentration-dependent decreases in cell viability, and the effects of CQ were temporally specific (B). Results represent mean ± SD obtained from at least three independent experiments. *$p<0.05$ vs. 0 μM vehicle CTL; $^{\#}p<0.05$ vs. 0-50 μM CQ (A), 0-36h (B), 0-15 μM ACQ (C), 0-50 μM HCQ (D) and 0-0.01 μM STS (E).

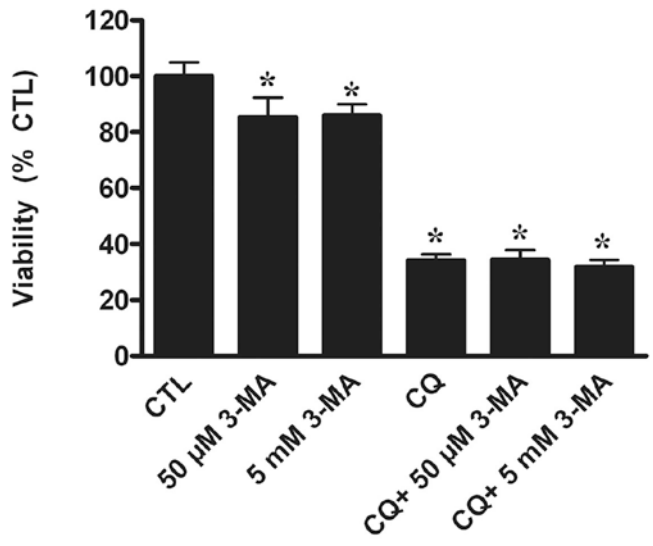


Pivtoraiko et al Supplemental Fig. 2

Supplemental Fig. 2. Low-dose bafilomycin attenuates stimulus-induced death of SH-SY5Y cells. 48h treatment with bafilomycin A1 (BafA1, 1 nM) significantly attenuates the reduction in cell viability following treatment with A) HCQ (50 µM) vs. ACQ (15 µM) or B) staurosporine (STS, 0.1 µM). *$p<0.05$ vs. 0 µM vehicle CTL; ** $p<0.05$ vs. HCQ; $^{\#}p<0.05$ vs. 0.1 µM STS; $^{\#\#}p<0.05$ vs. ACQ.

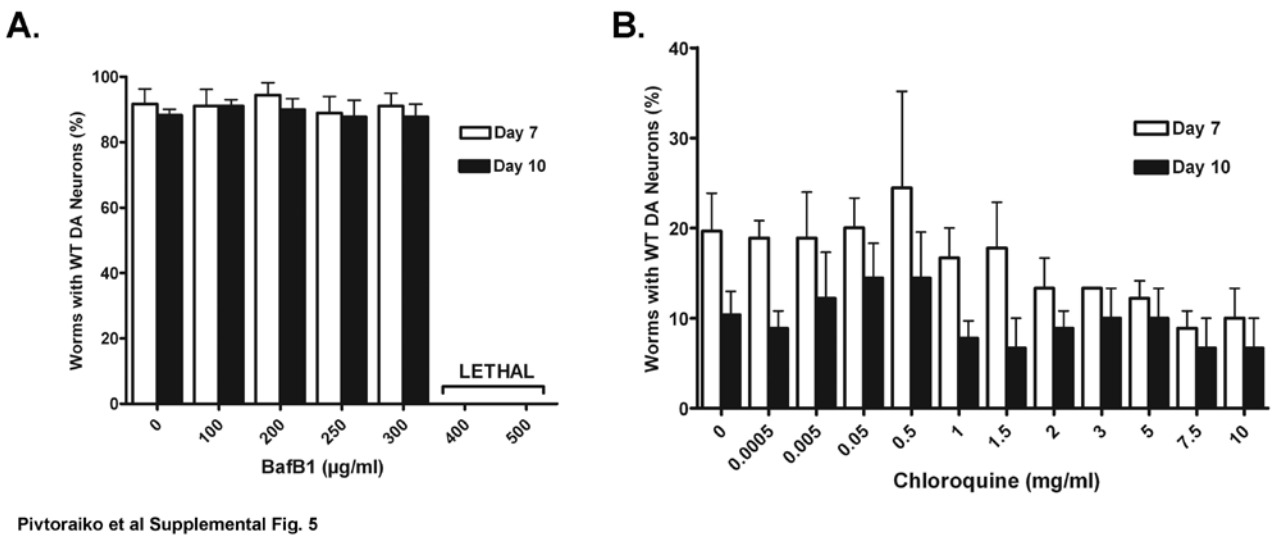


Supplemental Fig. 3. Low-dose bafilomycin attenuates chloroquine-induced cell death of differentiated SH-SY5Y cells. (A) Chloroquine (CQ, 50 μM) induces concentration-dependent decrease in viability of retinoic acid-differentiated SH-SY5Y cells, as measured by fluorogenic calcein conversion assay 48h after treatment. (B) Bafilomycin A1 (BafA1, 1 nM) significantly attenuates CQ-induced decrease in cell viability. Results represent mean ± SD obtained from at least three independent experiments. *$p<0.05$ vs. 0 μM vehicle CTL; **$p<0.05$ vs. CQ.



Pivtoraiko et al Supplemental Fig. 4

Supplemental Fig. 4. Inhibition of autophagy induction does not attenuate chloroquine-induced cell death. Differentiated SH-SY5Y cells were treated with either 50 µM or 5 mM 3-methyladenine (3-MA), a classical class-III PI3-K inhibitor, in the presence or absence of 50 µM chloroquine. 3-MA was added during the last 24h of the 48h incubation with 50 µM chloroquine to avoid additional 3-MA-specific toxicity. Viability was measured at 48h after chloroquine treatment. *$p<0.05$ vs. 0 µM vehicle CTL.



Pivtoraiko et al Supplemental Fig. 5

Supplemental Fig. 5. Effects of bafilomycin and chloroquine on DA neuron death in *C. elegans*. (A) Worms over-expressing GFP (but not α-syn) in DA neurons were acutely exposed to 0-500 µg/ml BafB1 for 24h and then scored for DA neuron loss as in Fig. 7 (A, B). Note that the 400-500 µg/ml concentrations of BafB1 were lethal to *C. elegans* embryos thus precluding neuron counts from these worms. (B) Worms over-expressing α-syn in DA neurons were crossed into worms mutant for *pgp-3*, a strain of worms that enhances CQ toxicity due to a predicted decrease in toxin export from cells. Worms were treated with 0.0005-10 mg/ml chloroquine and assessed for DA neuron loss as for BafB1 (Fig. 7 A, B). Treatment with chloroquine at any concentration did not alter the percentage of worms exhibiting WT DA neurons. Results represent mean ± SD obtained from at least three independent experiments, where 30 worms were analyzed for each experiment (n=90). *p<0.05 vs. 0 µg/ml vehicle CTL.

REFERENCES

Klionsky, D. J., Abeliovich, H., Agostinis, P. *et al* (11-21-2007) Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. *Autophagy.* 4 (2) 151-175.