

Low-dose bafilomycin attenuates neuronal cell death associated with autophagy–lysosome pathway dysfunction

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Abstract

We have shown previously that the plecomacrolide antibiotics bafilomycin A1 and B1 significantly attenuate cerebellar granule neuron death resulting from agents that disrupt lysosome function. To further characterize bafilomycin-mediated cytoprotection, we examined its ability to attenuate the death of naïve and differentiated neuronal SH-SY5Y human neuroblastoma cells from agents that induce lysosome dysfunction *in vitro*, and from *in vivo* dopaminergic neuron death in *C. elegans*. Low-dose bafilomycin significantly attenuated SH-SY5Y cell death resulting from treatment with chloroquine, hydroxychloroquine amodiaquine and staurosporine. Bafilomycin also attenuated the chloroquine-induced reduction in processing of cathepsin D, the principal lysosomal aspartic acid protease, to its mature 'active' form. Chloroquine induced autophagic vacuole accumulation and inhibited autophagic

flux, effects that were attenuated upon treatment with bafilomycin and were associated with a significant decrease in chloroquine-induced accumulation of detergent-insoluble α -synuclein oligomers. In addition, bafilomycin significantly and dose-dependently attenuated dopaminergic neuron death in *C. elegans* resulting from *in vivo* over-expression of human wild-type α -synuclein. Together, our findings suggest that low-dose bafilomycin is cytoprotective in part through its maintenance of the autophagy-lysosome pathway, and underscores its therapeutic potential for treating Parkinson's disease and other neurodegenerative diseases that exhibit disruption of protein degradation pathways and accumulation of toxic protein species.

Keywords: autophagy, bafilomycin, cathepsin D, lysosome, Parkinson's Disease, α -synuclein.

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The autophagy-lysosome pathway (ALP) is responsible for the highly-regulated recycling of intracellular contents, whereby macronutrients and damaged organelles are enclosed within double-membraned autophagic vacuoles (AVs) and delivered to lysosomes for pH-dependent degradation by lysosomal hydrolases. It is well known that intact lysosome function is critical for effective completion of the ALP (reviewed in (Pivtoraiko *et al.* 2009) and (Shacka *et al.* 2008). Lysosome dysfunction can potentially inhibit autophagy completion as demonstrated by robust AV accumulation followed by the induction of cell death (Zaidi *et al.* 2001); (Boya *et al.* 2005); (Shacka *et al.* 2006b). While the ALP is ordinarily cytoprotective, it is unclear whether AV accumulation resulting from lysosome dysfunction contributes to cell death.

Chloroquine is an anti-malarial drug and potent lysosomotropic agent. As a weak base chloroquine accumulates in

acidic vesicles and raises their pH (reviewed in Pivtoraiko *et al.* 2009). We and others have shown that chloroquine disrupts lysosome function and inhibits autophagy completion as demonstrated by AV accumulation, and induces both apoptotic and non-apoptotic cell death (Zaidi *et al.* 2001); (Boya *et al.* 2003); (Boya *et al.* 2005); (Shacka *et al.* 2006b); (Pivtoraiko *et al.* 2009).

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Abbreviations used: ALP, autophagy-lysosome pathway; AVs, autophagic vacuoles; CD, cathepsin D; DA, dopamine; FBS, fetal bovine serum; GFP, green fluorescent protein; mRFP, monomeric red fluorescent protein; PD, Parkinson's disease; tFLC3, tandem fluorescent-tagged LC3; V-ATPase, vacuolar-type ATPase; WT, wild-type.

Growing evidence suggests that the ALP is also altered in age-related neurodegenerative diseases including Parkinson's disease (PD), the most common neurodegenerative movement disorder. Alterations in autophagy were reported initially by the aberrant accumulation of AVs in substantia nigra neurons of PD patients (Anglade *et al.* 1997). Mutations in several PD-specific genes, including α -synuclein, LRRK2, Parkin and ATP13A2, are known to adversely affect autophagy and/or lysosome function (reviewed in Shacka *et al.* 2008 and Pan *et al.* 2008). α -Synuclein is a major component of Lewy bodies in PD brain and α -synuclein accumulation is thought to play an important causal role in the onset and progression of PD. Lysosomes are important for α -synuclein clearance, and cathepsin D (CD), the principal lysosomal aspartic acid protease, is the main lysosomal enzyme involved in the degradation of α -synuclein (Sevlever *et al.* 2008). Consistent with this hypothesis, CD deficiency has been reported to enhance α -syn toxicity (Qiao *et al.* 2008); (Cullen *et al.* 2009), and several studies indicate the therapeutic potential for autophagy induction in promoting α -synuclein clearance in PD (Webb *et al.* 2003); (Spencer *et al.* 2009); (Yang *et al.* 2009a); (Yu *et al.* 2009). Together, these findings suggest that ALP-targeted therapies may be effective in maintaining α -synuclein clearance and in general inhibiting neurodegenerative disease-associated neuropathology.

Bafilomycin A1 represents the plecomacrolide subclass of macrolide antibiotics and was characterized initially by its selective inhibition of vacuolar type-ATPase (V-ATPase) (Bowman *et al.* 1988). V-ATPase maintains the low pH of acidic vesicles through its regulation of proton pumping (Forgac 2007). At concentrations ≥ 10 nM, bafilomycin A1 inhibits V-ATPase and in turn increases intravesicular pH (Yoshimori *et al.* 1991), thus mimicking the effects of chloroquine. However, we have shown previously that the plecomacrolides bafilomycin A1, bafilomycin B1 and concanamycin all significantly attenuate chloroquine-induced death of cerebellar granule neurons (Shacka *et al.* 2006b); (Shacka *et al.* 2006a), at low concentrations (≤ 1 nM) which do not inhibit V-ATPase (Bowman *et al.* 1988) or induce AV accumulation (Shacka *et al.* 2006b). These data suggest that bafilomycin-mediated neuroprotection is independent of its inhibition of V-ATPase. However, whether bafilomycin affects the ALP or regulates α -synuclein clearance and neurotoxicity has not been investigated.

In the current study, we extended our analysis of chloroquine -induced death and bafilomycin A1 neuroprotection to SH-SY5Y, a human neuroblastoma cell line commonly used to model dopaminergic neurons. We also assessed the cytoprotective effects of bafilomycin in the nematode *Caenorhabditis elegans* that over-express human wild-type (WT) α -synuclein, which we have previously shown to induce both age- and dose-dependent neurodegeneration *in vivo* (Cao *et al.* 2005); (Hamamichi *et al.* 2008).

Our results indicate that bafilomycin attenuates neuronal cell death in all of these models consistent with its ability to maintain ALP function and reduce α -synuclein neurotoxicity.

Materials and methods

Cell culture

Naïve SH-SY5Y human neuroblastoma cells were cultured in Minimum Essential Medium Eagle (Cellgro, Herndon, VA, USA) and F12-K (ATCC, Manassas, VA, USA) medium supplemented with 0.5% sodium pyruvate, 0.5% non-essential amino acids (Cellgro), 1% penicillin/streptomycin (Sigma, St. Louis, MO, USA), and 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA). Cells were plated at 200 cells/mm² and grown for 24 h in media containing 1% FBS prior to treatment. Cells were treated from 0 to 48 h in media containing 1% FBS. SH-SY5Y cells were differentiated for 7–8 days in complete media supplemented with 10 μ M retinoic acid (Sigma). Retinoic-acid-supplemented media was replaced every 2–3 days. Unless otherwise noted, differentiated cells were plated at 400/mm² in differentiation media containing 2% B-27 (Invitrogen, Carlsbad, CA, USA) and were treated in this media for up to 48 h.

Cells were treated with chloroquine, hydroxychloroquine, amodiaquine, staurosporine or 3-methyladenine (Sigma), and either bafilomycin A1 (Sigma) or bafilomycin B1 (A.G. Scientific, San Diego, CA, USA).

Measurement of SH-SY5Y cell viability and Caspase 3-like activity

Cell viability was measured using calcein AM fluorogenic conversion assay (Invitrogen). Caspase 3-like activity was detected via fluorogenic DEVD cleavage assay and expressed relative to untreated controls. These assays were performed using previously published protocols (Nowoslawski *et al.* 2005).

Tandem fluorescent-tagged LC3 (tFLC3) assay of autophagic flux

Differentiated SH-SY5Y cells were transiently transfected with ptfLC3 (mammalian expression vector in plasmid DH5a; Entrez ID of insert U05784) (Addgene, Cambridge, MA, USA) using the Amaxa Nucleofector™ II device protocol (Lonza, Cologne, Germany). Two million cells were pelleted and resuspended in 100 μ L of Lonza™ Nucleofector reagent followed by addition of ptfLC3 DNA (2 μ g/reaction). Transfer of the reaction mixture was completed by electroporation in the Amaxa Nucleofector™ II device. Five hundred microlitre of neutralization media was immediately added followed by transfer of the full volume to a fresh tube, where cells recovered for 10 min, 22°C. Transfected cells were plated in poly-L-lysine coated glass chamber slides (LabTek, Rochester, NY, USA) at 120 000 cells/600 μ L media per well and kept at 37°C prior to treatment. Media was exchanged for fresh 4 h after plating to remove transfection reagents in addition to cells that did not survive the transfection reaction. Cells were treated with 50 μ M chloroquine in the presence or absence of 1 nM BafA1 for 8 h. After treatment slides were fixed with 3% paraformaldehyde for 45 min at 22°C, washed with 1 \times phosphate-buffered saline and cover-slipped. Cells were visualized using a Zeiss™ Observer.Z1 Laser Scanning Microscope (Thornwood, NY, USA) equipped with a Zeiss™ 40 \times 1.3 Oil DIC M27 Plan-Apochromat objective and

imaged using Zen™ 2008 LSM 710, V5.0 SP1.1 software. Fluorescence filters were used to observe bis benzemide (excitation 405 nm, emission 409–514 nm), GFP (excitation 488, emission 494–572 nm), and monomeric red fluorescent protein (mRFP) (excitation 543, emission 585–734).

Generation and analysis of *C. elegans*

Isogenic *C. elegans* strain UA44 (*baln11*; $P_{dat-1}::\alpha\text{-syn}$, $P_{dat-1}::\text{GFP}$) that co-express α -synuclein and green fluorescent protein in dopamine neurons and exhibit age-dependent α -synuclein-induced neurodegeneration were age synchronized by bleaching as previously described (Lewis and Fleming 1995) and placed in 1 mL of water containing 5% methanol (with or without bafilomycin B1) for 24 h at 20°C with gentle agitation. After incubation, worms were washed with M9 buffer thrice, transferred to nematode growth media plates, and grown at 20°C. For each trial, 30 worms were immobilized with 3 mM levamisole, transferred onto a 2.5% agarose pad, and analyzed for neuroprotection at both day 7 and day 10 post-hatchings. Worms were considered WT when there were four intact cephalic type DA neurons and two anterior deirid type dopamine (DA) neurons without any signs of degeneration. Each bafilomycin B1 treatment was analyzed in triplicate (90 worms per concentration).

To determine if increased concentrations of bafilomycin B1 induced DA neuron cell death, the *C. elegans* strain BY200 ($P_{dat-1}::\text{GFP}$) (Nass and Blakely 2003), which express GFP in the DA neurons without degeneration, was synchronized, treated with high concentrations of bafilomycin B1, and analyzed as described above. To study the effect of chloroquine on DA neurons, *C. elegans* strain UA44 was crossed into knockout strain NL131 [*pgp-3(pk18)*], shown previously to be sensitive to chloroquine (Broeks *et al.* 1995) to generate the isogenic strain UA146 [*baln11*; *pgp-3(pk18)*]. This strain was synchronized, treated with chloroquine using methods similar to bafilomycin B1, and analyzed as described above.

Statistics

Significant effects of treatment were analyzed either by one-factor ANOVA (three or more groups) or by unpaired, two-tailed *t*-test (two groups). *Post hoc* analysis was conducted using Bonferroni's test. A level of $p < 0.05$ was considered significant.

Results

Bafilomycin B1 is a structural analog of bafilomycin A1 and both compounds were shown previously at high doses and with equal potency to inhibit V-ATPase (Bowman *et al.* 1988), whereas we have shown that low doses of each are equally effective in attenuating chloroquine-induced death of cerebellar granule neurons (Shacka *et al.* 2006b). Treatment of naïve SH-SY5Y cells for 48 h with bafilomycin A1 (Fig. 1a) or bafilomycin B1 (Fig. 1b) did not alter cell viability when added alone at ≤ 1 nM, whereas both significantly reduced cell viability at concentrations ≥ 6 nM, effects similar to those reported in cerebellar granule neurons (Shacka *et al.* 2006a,b). To determine if bafilomycin-induced death of SH-SY5Y cells was apoptotic,

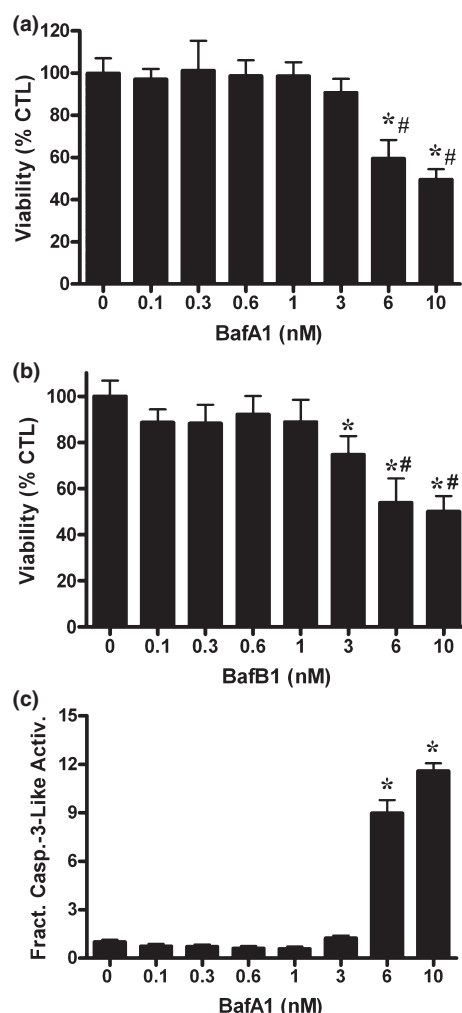


Fig. 1 Low-dose bafilomycin is not cytotoxic to SH-SY5Y cells. Forty eight hour treatment with bafilomycin A1 (BafA1, a) or bafilomycin B1 (BafB1, b) decreases cell viability at concentrations ≥ 6 nM for BafA1 and ≥ 3 nM for BafB1. (c) BafA1 significantly increases caspase 3-like activity at concentrations ≥ 6 nM. Results represent mean \pm SD from at least three independent experiments. * $p < 0.05$ versus 0 μM vehicle CTL; # $p < 0.05$ versus 0–3 nM BafA1 (a) or 0–1 nM BafB1 (b).

caspase 3-like activity was measured following treatment for 48 h with 0.1–10 nM bafilomycin A1 (Fig. 1c). Significant increases in caspase 3 like activity were observed only at bafilomycin A1 concentrations ≥ 6 nM, suggesting that cell death induced by high concentrations of bafilomycin A1 (Fig. 1a) was apoptotic. These results confirm that bafilomycin A1 and bafilomycin B1 neither induced SH-SY5Y cell death or apoptosis at concentrations ≤ 1 nM.

Naïve SH-SY5Y cells were treated with cell death stimuli 24 h after plating, and cell viability was measured 48 h after treatment (Figure S1). Chloroquine significantly decreased cell viability at concentrations ≥ 20 μM (Figure S1a), and cell death was maximal at 60–80 μM . Upon treatment of cells with 50 μM chloroquine, significant decreases in cell

viability were observed from 24 to 48 h following treatment (Figure S1b), and was maximal at 48 h. The chloroquine analogs amodiaquine (Figure S1c) and hydroxychloroquine (Figure S1d) significantly reduced cell viability in a concentration-dependent manner similar with that of chloroquine, although amodiaquine was 2–3 times more potent in reducing cell viability by 50% (~15 μM) in comparison with chloroquine (~40 μM) and hydroxychloroquine (~50 μM). In addition, treatment for 48 h with staurosporine, a classical apoptotic stimulus shown previously to disrupt lysosome function (Bidere *et al.* 2003); (Kagedal *et al.* 2005), significantly decreased SH-SY5Y cell viability at all concentrations tested (0.005–0.1 μM), and was maximally effective at 0.1 μM (Figure S1e).

When bafilomycin A1 or bafilomycin B1 were added at 0.1–1 nM concurrently for 48 h with 50 μM chloroquine, we observed significant attenuation of chloroquine-induced cell death (Fig. 2a and b), an effect that was maximal at 0.6–1 nM for bafilomycin A1 (Fig. 2a) and 0.3–1 nM for bafilomycin B1 (Fig. 2b). Bafilomycin A1 (1 nM) also significantly attenuated the loss in cell viability following 48 h treatment with amodiaquine (15 μM) or hydroxychloro-

quine (50 μM ; Figure S2a) or staurosporine (0.1 μM , Figure S2b). High-dose bafilomycin has been shown previously to directly inhibit chloroquine localization within lysosomes (Boya *et al.* 2003). To rule out this potential interaction of low-dose bafilomycin against chloroquine-induced cell death, cells were pre-treated with bafilomycin A1 for either 12 h (Fig. 2c) or 24 h (Fig. 2d) and following its wash-out post-treated with 50 μM chloroquine for 48 h. Both 12 h and 24 h pre-treatment with bafilomycin A1 significantly attenuated cell death induced by chloroquine post-treatment, supporting our argument that the cytoprotective effects of low-dose bafilomycin are not from its inhibition of chloroquine localization to lysosomes.

We have shown previously that chloroquine induces robust neuron apoptosis (Zaidi *et al.* 2001); (Shacka *et al.* 2006b) and that *bax* deficiency significantly attenuates chloroquine-induced death of cultured neurons, suggesting the importance of the intrinsic apoptotic pathway in regulating chloroquine-induced death (Zaidi *et al.* 2001); (Shacka *et al.* 2006b). We have also shown that low-dose bafilomycin attenuates chloroquine-induced apoptosis of cultured neurons (Shacka *et al.* 2006b). We next sought to determine the

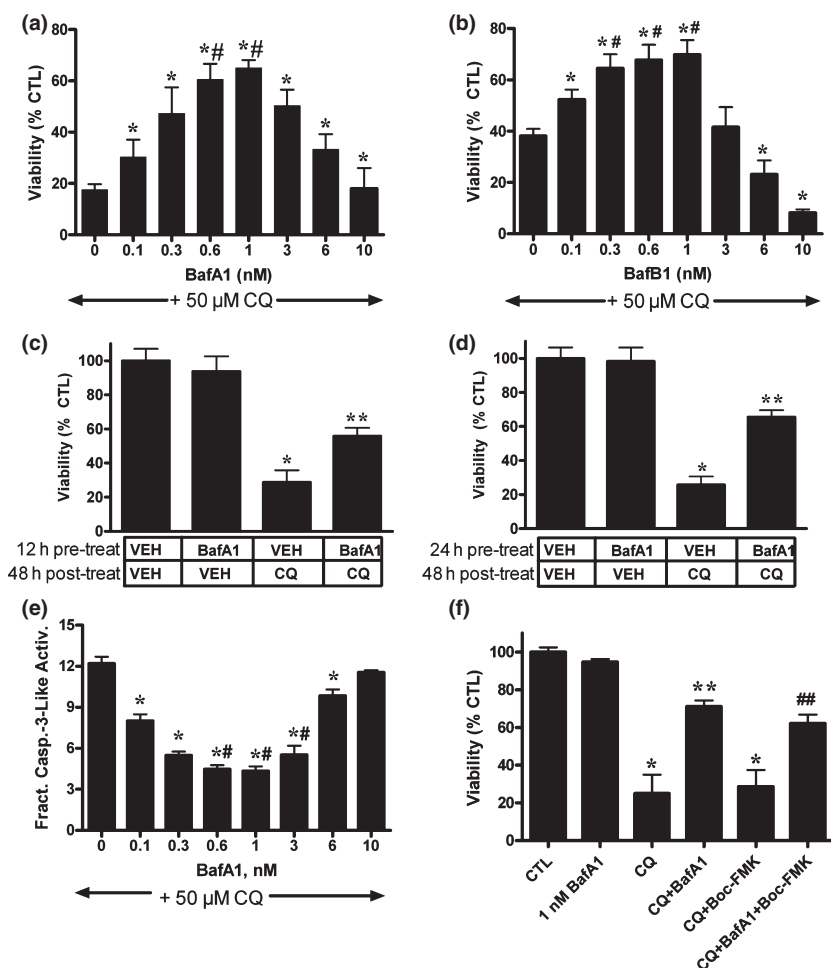


Fig. 2 Low-dose bafilomycin attenuates chloroquine-induced cell death and apoptosis. Forty eight hour treatment with bafilomycin A1 (BafA1, a) or bafilomycin B1 (BafB1, b) significantly attenuates chloroquine (CQ, 50 μM)-induced cell death from 0.1 to 6 nM for BafA1 (a) and from 0.1 to 1 nM for BafB1 (b). (c–d) Pre-treatment with 1 nM BafA1 for either 12 h (c) or 24 h (d) significantly attenuates the reduction in viability following 48 h post-treatment with 50 μM CQ. (e) Twenty four hour treatment with BafA1 (1 nM) significantly attenuates CQ-induced increase in caspase 3-like activity. (f) Co-treatment with the general caspase inhibitor BOC-Asp (OMe)-FMK (Boc-FMK, 30 μM) neither attenuates CQ-induced cell death nor enhances the cytoprotective effects of BafA1 against CQ-induced cell death. * $p < 0.05$ versus 0 μM vehicle CTL; ** $p < 0.05$ versus vehicle pre-treatment/CQ post-treatment (c–d) or CQ (F); # $p < 0.05$ versus 0–3 nM and 3–6 nM BafA1 (a), 0–0.1 nM and 3–10 nM BafB1 (b), 0–0.1 nM and 6–10 nM BafA1 (c); ## $p < 0.05$ versus CQ + Boc-FMK.

effects of chloroquine \pm low-dose bafilomycin A1 on the induction of SH-SY5Y apoptosis. Treatment for 24 h with 50 μ M chloroquine significantly induced apoptosis as measured by enzymatic caspase 3-like activity (Fig. 2e), an effect that was significantly attenuated by treatment with bafilomycin A1 and was maximally protective at 0.3–3 nM. To determine if caspase activation was a commitment point for chloroquine-induced death of SH-SY5Y cells, we measured the effects of chloroquine \pm low-dose bafilomycin A1 on cell viability in the presence of the general caspase inhibitor BOC-Asp (OMe)-FMK (Fig. 2f). This caspase inhibitor at 30 μ M completely blocked chloroquine-induced caspase 3 activity but neither attenuated chloroquine-induced cell death nor enhanced bafilomycin-mediated cytoprotection. Together these data obtained in SH-SY5Y cells corroborate our previous findings in cerebellar granule neurons (Shacka *et al.* 2006b); (Shacka *et al.* 2006a) suggesting that: (i) bafilomycins potently attenuate cell death and apoptosis induced by chloroquine and structurally similar analogs when used at concentrations \leq 1 nM; and (ii) bafilomycin regulation of chloroquine-induced cell death occurs upstream of caspase activation.

We have shown recently that chloroquine-induced death of SH-SY5Y cells correlated with a compromise in the maturation of CD (Pivtoraiko *et al.* 2009). Treatment for 24 h with 50 μ M chloroquine significantly induced levels of the unprocessed, 50 kDa pre-pro form of CD (Fig. 3a and b) and significantly decreased levels of the mature active 32 kDa form of CD (Fig. 3a and d). These effects were observed as early as 12 h following chloroquine treatment (data not shown). The 47 kDa pro-form of CD was not significantly affected by treatment (Fig. 3c). When added by itself, 1 nM bafilomycin A1 did not alter CD processing. However, 1 nM bafilomycin A1 significantly attenuated the chloroquine-induced decrease in the mature form of CD (Fig. 3d), suggesting the ability of bafilomycin A1 to partially recover lysosomal enzyme function at concentrations that significantly attenuates chloroquine-induced cell death.

To determine if neuronal differentiated SH-SY5Y cells exhibited protective effects of bafilomycin against chloroquine-induced cell death similar with that of naïve undifferentiated cells, naïve SH-SY5Y cells were differentiated for 7–8 days with retinoic acid. Chloroquine induced a concen-

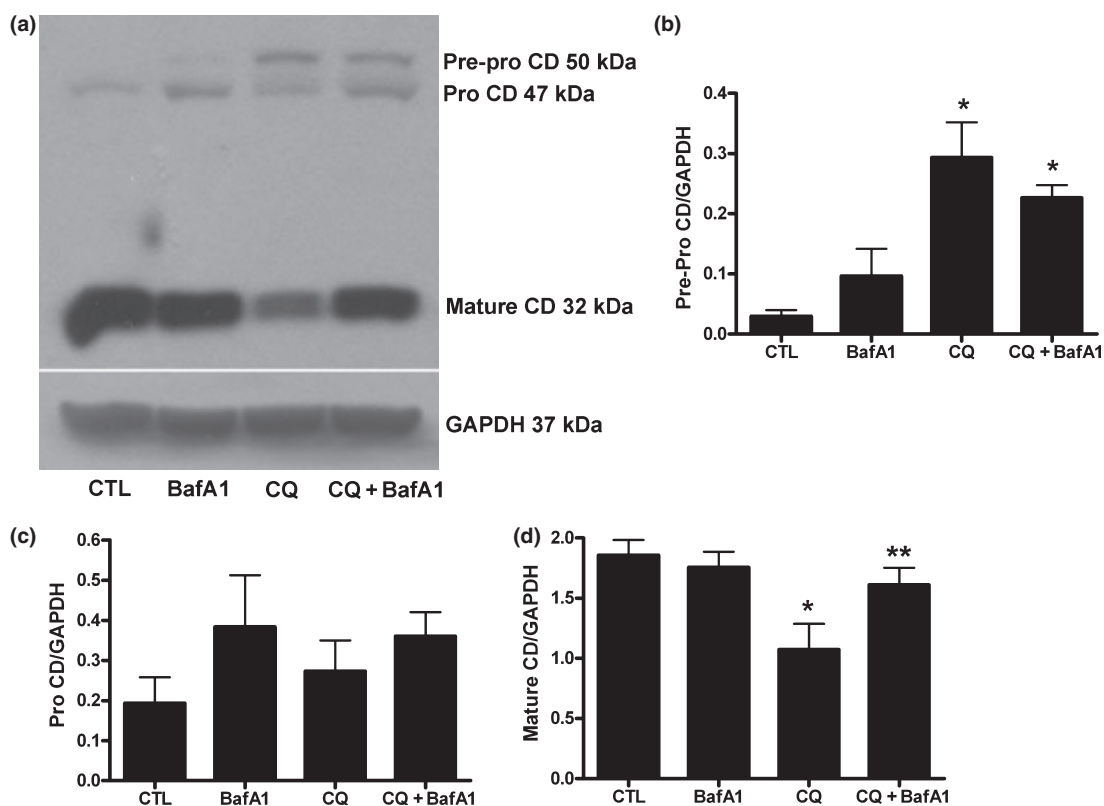


Fig. 3 Bafilomycin A1 attenuates chloroquine-induced inhibition of cathepsin D (CD) processing. (a) Whole cell lysates of SH-SY5Y cells were collected 24 h following treatment with chloroquine (CQ, 50 μ M) and/or bafilomycin A1 (BafA1, 1 nM), and subjected to western blot analysis for CD (pre-pro form, 50 kDa; pro form, 47 kDa; mature 'ac-

tive' form, 32 kDa). Blots were stripped and re-probed for GAPDH (37 kDa) loading control. Levels of (b) pre-pro CD, (c) pro CD and (d) mature CD were normalized to levels of β -tubulin. Results represent mean \pm SD from at least three independent experiments. * p < 0.05 versus 0 μ M vehicle CTL; ** p < 0.05 versus CQ.

tration-dependent decrease the viability of differentiated cells (Figure S3a) in differentiated cells similar with that of naïve cells (Figure S1a), an effect that was significantly attenuated by the addition of 1 nM bafilomycin A1 (Figure S3b). We next sought to determine if chloroquine affects the clearance of endogenous α -synuclein oligomers in neuronal SH-SY5Y cells, as it is well known that intact lysosome function plays an important role in α -synuclein degradation (Lee *et al.* 2004); (Qiao *et al.* 2008); (Sevlever *et al.* 2008); (Cullen *et al.* 2009). Triton X-soluble versus insoluble fractions was prepared from lysates of differentiated SH-SY5Y cells following 48 h treatment with chloroquine (50 μ M) in the presence or absence of low-dose bafilomycin A1 (1 nM). Treatment with chloroquine significantly increased levels of endogenous detergent-insoluble α -synuclein oligomers (Fig. 4a and b). By itself, 1 nM bafilomycin A1 did not significantly alter levels of insoluble α -synuclein oligomers (Fig. 4a and b). In contrast, 1 nM bafilomycin A1 significantly attenuated the chloroquine-induced increase in detergent-insoluble α -synuclein oligomers present in the detergent-insoluble fraction (Fig. 4a and b). Chloroquine and/or bafilomycin had no effect on endogenous levels of

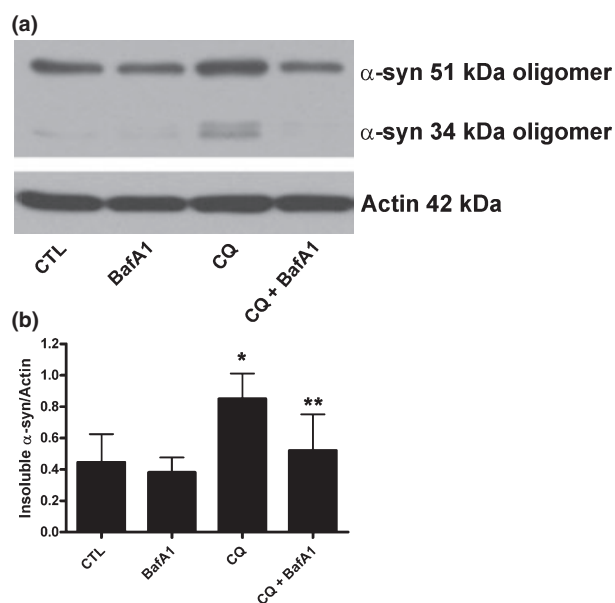


Fig. 4 Bafilomycin A1 attenuates chloroquine-induced increase in detergent-insoluble endogenous α -syn oligomers. (a) Representative western blot analysis of endogenous α -syn high molecular weight oligomers (34 and 51 kDa) in detergent-insoluble fractions, prepared from lysates of differentiated SH-SY5Y cells collected 48 h after treatment with chloroquine (CQ, 50 μ M) and/or bafilomycin A1 (BafA1, 1 nM). Blots were stripped and re-probed for actin (42 kDa) loading control. (b) Levels of insoluble α -syn high molecular weight oligomers were quantified and normalized to levels of actin. Results represent mean \pm SD from five independent experiments. * $p < 0.05$ versus 0 μ M vehicle CTL; ** $p < 0.05$ versus CQ.

detergent-soluble α -synuclein (data not shown). Together, these data indicate that low-dose bafilomycin regulates the clearance of detergent-insoluble forms of endogenous α -synuclein oligomers.

We have shown previously that chloroquine-induced death of cerebellar granule neurons was accompanied by the robust accumulation of AVs (Shacka *et al.* 2006b). To determine the effects of chloroquine \pm low-dose bafilomycin on AV accumulation in neuronally differentiated SH-SY5Y cells, western blot analysis was performed on detergent-soluble versus insoluble protein fractions (Fig. 5). Levels of LC3-II, which were used as a selective marker of AVs, were not increased following treatment with bafilomycin A1 (1 nM) or in vehicle-treated control cells. Treatment with chloroquine (50 μ M) for 48 h significantly induced AV accumulation as measured by LC3-II/actin ratios versus control-treated cultures (Fig. 5a–c). In contrast, co-treatment with chloroquine and bafilomycin A1 significantly decreased AV accumulation compared with chloroquine treatment alone (Fig. 5a–c).

To further investigate whether attenuation of chloroquine-induced death by low-dose bafilomycin was associated with alterations in ALP function we utilized the tFLC3 assay (Fig. 6), an *in vitro* fluorescence measure of autophagic flux (Kimura *et al.* 2007); (Kimura *et al.* 2009). Over-expression of the tFLC3 plasmid results in tandem expression of both mRFP-LC3 and GFP-LC3. Under basal conditions, GFP-LC3 loses fluorescence because of the acidic nature of lysosomes. However, a compromise in lysosome stabilizes GFP-LC3 fluorescence and in turn increases GFP-LC3 and mRFP-LC3 co-localization. Cultures of tFLC3-transfected cells were treated for 8 h with chloroquine (50 μ M) in the presence or absence of 1 nM bafilomycin A1. Both vehicle control and low-dose bafilomycin-treated cells exhibited basal RFP and GFP fluorescence and little if any co-localization (Fig. 6a and b). Treatment with chloroquine induced the appearance of both GFP-LC3 and RFP-LC3 fluorescent punctae and their apparent co-localization, (Fig. 6c), effects that appeared to be dramatically reduced in chloroquine plus bafilomycin A1-treated cultures (Fig. 6d). Together, these data suggest that low dose bafilomycin may preserve lysosome function and thus, maintenance of autophagic flux following chloroquine exposure.

We also attempted to determine if inhibition of autophagy induction by treatment of cultures with 3-methyladenine, an inhibitor of class-III PI3-K, attenuated chloroquine-induced death. Treatment of differentiated SH-SY5Y cells with either 50 μ M or 5 mM 3-methyladenine did not attenuate chloroquine-induced death. However, 3-methyladenine was toxic in its own right to SH-SY5Y cells compared with vehicle control (Figure S4), suggesting the importance of basal autophagy induction in promoting cell survival. These data suggest that autophagy

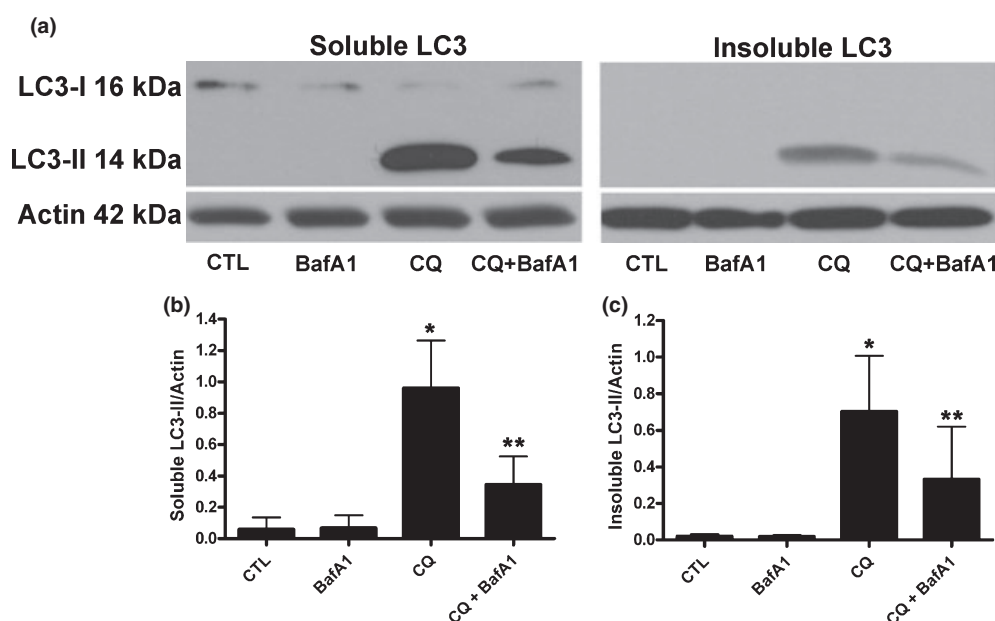


Fig. 5 Bafilomycin A1 attenuates chloroquine-induced autophagic vacuoles accumulation. (a) Representative western blot analysis for LC3-I (cytosolic, 16 kDa) versus LC3-II (autophagic vacuole membrane-specific, 14 kDa) in detergent-soluble (LEFT) versus -insoluble (RIGHT) fractions prepared from differentiated SH-SY5Y cells 48 h after treatment with chloroquine (CQ, 50 μ M) and/or bafilomycin A1

(BafA1, 1 nM). Blots were stripped and re-probed for actin (42 kDa) loading control. Levels of LC3-II were quantified and normalized to actin for detergent-soluble (b) and detergent-insoluble (c) fractions. Note only detergent soluble fractions exhibited LC3-I immunoreactivity. Results represent mean \pm SD from at least four independent experiments. * p < 0.05 versus 0 μ M vehicle CTL; ** p < 0.05 versus CQ.

induction is not important for regulating chloroquine-induced cell death.

To determine if plecomacrolides protected against α -synuclein-induced DA neurodegeneration *in vivo*, isogenic worms over-expressing human WT α -synuclein in dopamine neurons were acutely exposed to bafilomycin B1 for 24 h during larval development, and subsequently scored for dopaminergic neuron loss at either 7 or 10 days post-hatching (4 and 7 day adults, respectively). Acute exposure of animals to 50–150 μ g/mL bafilomycin B1 showed significant protection against α -synuclein-induced dopaminergic degeneration (Fig. 7). Exposure of these worms to lower concentrations of bafilomycin B1 did not significantly protect against dopaminergic neuron degeneration at day 7 (Fig. 7a), whereas higher concentrations induced DA neuron death in a majority of the animals. At day 10, animals exposed to either 50 or 100 μ g/mL bafilomycin B1 still exhibited significant protection against neurodegeneration (Fig. 7b). Worms lacking α -syn over-expression were also treated with bafilomycin B1 and the percentage of worms exhibiting DA neurons at either 7 or 10 days following treatment was not significantly different from vehicle control at concentrations ranging from 100 to 300 μ g/mL (Figure S5a). However, treatment of worms with 400–500 μ g/mL bafilomycin B1 was lethal to the embryos, thus precluding neuron counts from these worms

and suggesting the importance of intact V-ATPase function for worm survival. Together these results provide *in vivo* evidence that acute exposure of bafilomycin protects dopaminergic neurons against α -synuclein-induced neurodegeneration.

As it is possible that low-dose bafilomycin exerted a 'pre-conditioning effect' on DA neuron survival, we sought out to determine if treatment with low doses of chloroquine also protected against DA neuron death in worms. However, as worms are naturally resistant to some natural toxins, including chloroquine (Broeks *et al.* 1995), we crossed α -synuclein over-expressing worms into those mutant for *pgp-3*. PGP-3 encodes a P-glycoprotein transmembrane protein that has been predicted to export toxins such as chloroquine from cells and thus increase resistance to chloroquine toxicity (Broeks *et al.* 1995). Worms were treated with eleven concentrations of chloroquine ranging from 0.0005 to 10 mg/mL (as with bafilomycin-treated worms) and assayed for protection against α -synuclein-induced DA neurodegeneration (Figure S5b). Significant differences between treated or non-treated DA neurons were not observed at day 7 or 10, although there was a general trend of DA neurodegeneration with the highest concentrations of chloroquine. These results suggest that bafilomycin is specific in exerting a possible pre-conditioned effect in attenuating α -synuclein-induced cytotoxicity.

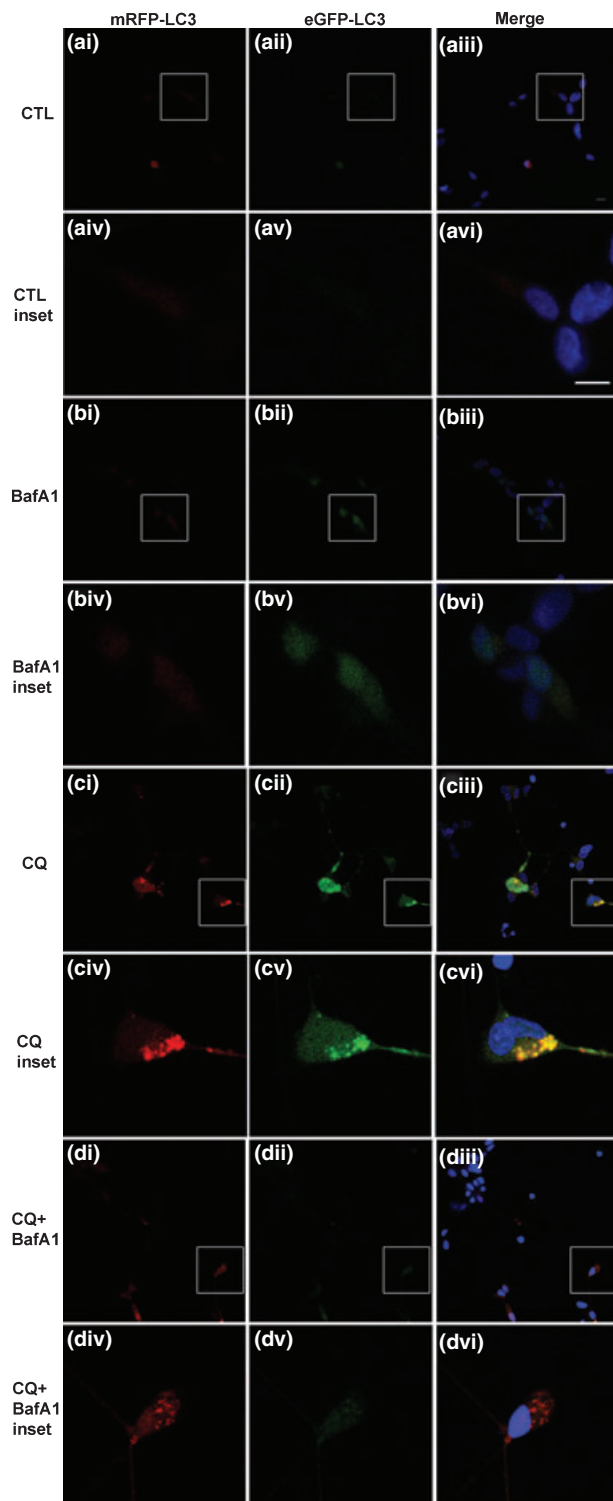


Fig. 6 Bafilomycin A1 attenuates chloroquine-induced inhibition of autophagic flux. Differentiated SH-SY5Y were transiently transfected to over-express tandem fluorescently-tagged LC3 and following 24 h recovery were treated for 8 h with chloroquine (CQ, 50 μ M) and/or bafilomycin A1 (BafA1, 1 μ M) to observe effects of low-dose BafA1 on chloroquine-induced co-localization (merged image, right panel) of mRFP-LC3 (left panels) and eGFP-LC3 (center panels) fluorescent punctae, suggesting inhibition of autophagic flux. Cells were fixed and imaged via confocal microscopy as described in the Methods section. Panels i–iii from CTL (a), BafA1 (b), CQ (c) and CQ+BafA1 are low magnification images; the box in each of these panels indicates higher magnification inset panels iv–vi for each. Images are representative of three independent experiments. Scale bar in a-iii and a-vi = 10 μ M.

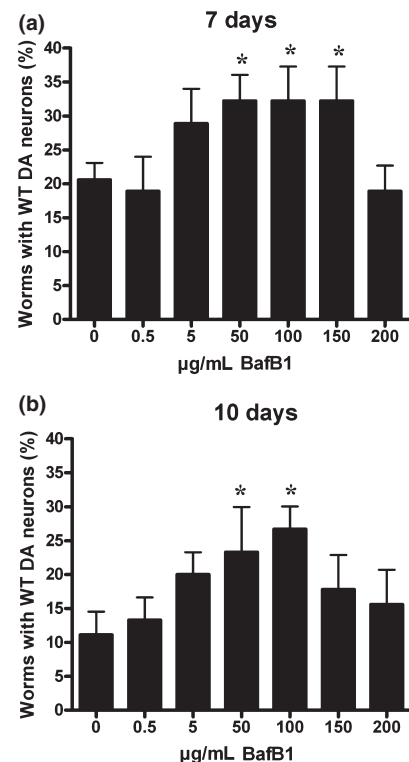


Fig. 7 Bafilomycin attenuates the death of DA neurons in *C. elegans* following over-expression of wild-type (WT) human α -syn. (a, b) Worms over-expressing α -syn in DA neurons were acutely exposed to bafilomycin B1 (BafB1, 0–200 μ g/mL) for 24 h during larval development, and then subsequently scored for DA neuron loss at either 7 days (a) or 10 days (b) post-hatching (4 and 7 day adults, respectively). Results represent mean \pm SD from at least three independent experiments, where 30 worms were analyzed for each experiment ($n = 90$). * $p < 0.05$ versus 0 μ g/mL vehicle CTL.

Discussion

The function of bafilomycins was defined originally by its selective inhibition of V-ATPase (Bowman *et al.* 1988), which effectively increases the pH of acidic vesicles.

Bafilomycin-mediated inhibition of V-ATPase results in the inhibition of lysosomal enzyme function and/or processing (Ishidoh and Kominami 2002); (Singh *et al.* 2006), induction of lysosome membrane permeabilization (Nakashima *et al.* 2003), inhibition of AV-lysosome fusion (Yoshimori *et al.* 1991) as well as potent inhibition of

macroautophagy completion followed by induction of cell death (Shacka *et al.* 2006b). In contrast, results of the present study suggest that bafilomycins significantly attenuate neuronal cell death caused by agents that disrupt lysosome function and by over-expression of WT human α -synuclein, when used at concentrations that do not inhibit V-ATPase (Bowman *et al.* 1988) or affect vesicular pH (Shacka *et al.* 2006b). Importantly, the cytoprotective concentrations of bafilomycins used in our study (≤ 1 nM) do not disrupt the ALP when used alone, and actually attenuate markers of ALP dysfunction (decrease in CD maturation and AV accumulation; inhibition of autophagic flux; increase in detergent-insoluble α -synuclein) caused by the lysosomotropic agent chloroquine. Together, these findings delineate a potentially novel mechanism of action for bafilomycins as ALP preservation agents, and may serve to identify future therapeutics capable of delaying the onset and/or progression of neurodegenerative diseases including PD.

Bafilomycin A1 significantly and dose-dependently attenuated SH-SY5Y cell death induced by the lysosomotropic agent's chloroquine, hydroxychloroquine or amodiaquine (Fig. 2; Figure S2). Amodiaquine exhibited a three-fold higher potency than chloroquine and hydroxychloroquine (Figure S1), a disparity documented previously in other cell lines (Basque *et al.* 2008) and may be as a result of structural differences in amodiaquine (phenolic substitution in its side chain) and/or its known uptake by an active transporter (Hayeshi *et al.* 2008). In addition, both bafilomycin A1 and bafilomycin B1 provided similar concentration-dependent protection against chloroquine-induced cell death (Fig. 2). Cytoprotective concentrations of bafilomycin A1 (≤ 1 nM) neither induced AV accumulation (Fig. 5) nor affected CD processing (Fig. 3). The effects of low-dose bafilomycin are similar to those found previously in cerebellar granule neurons, which indicated a lack of effect on pH-dependent lysotracker red fluorescence and AV accumulation (Shacka *et al.* 2006b).

Low-dose bafilomycin also significantly attenuated staurosporine-induced cell death (Figure S2), although its relative protection was modest in comparison with that of chloroquine. Staurosporine has been shown to induce lysosome dysfunction in different cell types (Bidere *et al.* 2003); (Kagedal *et al.* 2005), as indicated by lysosomal membrane permeabilization, an increase in cytosolic cathepsins and induction of apoptosis. Staurosporine's effects on lysosome function are most likely indirect compared with chloroquine, and staurosporine has been shown previously to disrupt several different protein kinase signaling pathways (Ruegg and Burgess 1989). These reasons may explain why bafilomycin-mediated cytoprotection is less robust against staurosporine versus chloroquine and other lysosomotropic agents. Our previous investigations indicated that low-dose bafilomycin did not attenuate staurosporine-induced death of

cerebellar granule neurons (Shacka *et al.* 2006b). This discrepancy may be as a result of subtle cell-type-specific differences in the timing of and/or sensitivity to staurosporine-induced cell death.

The general caspase inhibitor BOC-Asp (OMe)-FMK neither attenuated chloroquine-induced cell death nor enhanced the attenuation of chloroquine-induced cell death by bafilomycin A1 (Fig. 2f), at a concentration that completely inhibited chloroquine-induced caspase 3-like activity. Thus bafilomycin A1 may attenuate chloroquine-induced cell death at a point either upstream and/or independent of caspase 3 activation. Our previous studies in cerebellar granule neurons also demonstrated that chloroquine-induced cell death did not require caspase 3 activation or expression, but was attenuated by the targeted deletion of *bax*, which regulates apoptosis upstream of caspase activation (Shacka *et al.* 2006b). However, *Bax* deficiency did not further attenuate chloroquine-induced cell death upon treatment with bafilomycin A1, suggesting that bafilomycin A1 may regulate both *Bax*-dependent and -independent cell death pathways resulting from lysosome dysfunction.

The nematode *C. elegans* is an useful model organism to study neurodegeneration *in vivo* caused by either chemical (Nass *et al.* 2002); (Cao *et al.* 2005) or genetic factors (Cooper *et al.* 2006); (Hamamichi *et al.* 2008). In addition, *C. elegans* contains only eight readily identified dopaminergic neurons, six in the anterior (two pairs of cephalic and one pair of anterior deirid) and two in the posterior body segments (one pair of posterior deirid), making them a powerful model to study dopaminergic neuron degeneration in particular, allowing an unprecedented level of accuracy in quantifying effects of modifiers. Bafilomycin B1 significantly attenuated dopaminergic neuron death in *C. elegans* following over-expression of WT human α -synuclein (Fig. 7) where an inverted 'U' shaped dose response curve was observed 10 days after initial treatment. The maximal protective concentration of bafilomycin B1 in *C. elegans* was approximated at 100 μ g/mL or 161 μ M, a 160-fold higher concentration for optimal cytoprotection (1 nM) in cultured cells (Fig. 3); (Shacka *et al.* 2006b); (Shacka *et al.* 2006a). However, *C. elegans* has a protective cuticle layer that most likely compromised bafilomycin diffusion and penetration, an effect that is well characterized for other compounds (Rand and Johnson 1995; Holden-Dye and Walker 2007). In addition, the amount of active bafilomycin capable of affecting dopaminergic neurons in *C. elegans* may be further lowered upon metabolism within the worm, as it has been demonstrated previously with other compounds (Rand and Johnson 1995), thus necessitating a higher effective concentration range than optimal for cultured cells. Over-expression of WT *ATP13A2*, a gene expressing a lysosomal ATPase and mutations in which are associated with a juvenile-onset hereditary parkinsonism (Klein and Lohmann-Hedrich 2007), attenuates neuron death induced by α -synuclein

over-expression in *C. elegans* (Gitler *et al.* 2009), further implicating the importance of intact lysosome function in regulating α -synuclein-induced neurotoxicity. Thus, it is conceivable that bafilomycin B1 protected against over-expression of WT α -synuclein in *C. elegans* in part through its preservation of lysosomal function and promotion of α -synuclein clearance. Importantly, results in *C. elegans* suggest that bafilomycin attenuates dopaminergic neuron death following a stimulus (α -synuclein over-expression) that is, on one hand distinct from treatment with lysosomotropic agents *in vitro* yet may produce the same end result (disruption of the ALP).

Chloroquine treatment increased levels of high molecular weight, oligomeric forms of endogenous detergent-insoluble α -synuclein (Fig. 4), an effect that was significantly attenuated by low-dose bafilomycin A1. Aggregated α -synuclein is the most abundant protein composing Lewy bodies in PD, dementia with Lewy bodies, and a Lewy body variant of Alzheimer's disease (Trojanowski and Lee 1998). Whether α -synuclein oligomerization and aggregation are cytotoxic or cytoprotective is controversial, and evidence for both has been suggested (Hasegawa *et al.* 2004); (Rochet *et al.* 2004); (Ruan *et al.* 2009); (Yang *et al.* 2009b); (Yu *et al.* 2009). However, it is generally accepted that enhanced α -synuclein clearance is cytoprotective (Yu *et al.* 2009). The ALP and the ubiquitin-proteasomal system are both involved in α -synuclein clearance (Webb *et al.* 2003); (Lee *et al.* 2004); (Vogiatzi *et al.* 2008) and alterations in both are associated with α -synuclein aggregation in PD brain (Chu *et al.* 2009). While the relative importance of the ALP versus the ubiquitin-proteasomal system on α -synuclein clearance under normal physiological conditions and during a pathological process such as PD is not completely understood, recent data suggest that ALP inhibition has a more profound role in accumulation of WT α -synuclein (Vogiatzi *et al.* 2008) and that these distinct protein degradation mechanisms can act in a compensatory manner (Pandey *et al.* 2007). It has also been shown that CD is the main lysosomal protease responsible for α -synuclein degradation (Sevlever *et al.* 2008) and that α -synuclein aggregation and toxicity are significantly impacted by relative expression levels of CD (Qiao *et al.* 2008). In SH-SY5Y cells, attenuation of chloroquine-induced cell death by bafilomycin A1 was associated with a partial restoration of mature CD and a decrease in endogenous, oligomeric detergent-insoluble α -synuclein. Together, our data suggest that the bafilomycin cytoprotection is mediated, at least in part, by preventing accumulation of potentially toxic oligomeric detergent-insoluble α -synuclein forms through restoration of the ALP.

Additional evidence supporting the ability of low-dose bafilomycin to 'preserve' ALP function following chloroquine treatment is indicated by its ability to attenuate the chloroquine-induced decrease in the mature, 'active' form of CD (Fig. 3), decrease in the chloroquine-induced accu-

mulation of AVs (Fig. 5) and attenuate the chloroquine-induced inhibition of autophagic flux (Fig. 6). CD is synthesized on rough endoplasmic reticulum and undergoes initial processing in the Golgi, in particular from the pre-pro to pro forms. However, the mature form of CD is generated from proteolytic cleavage in the low pH environment of lysosomes (Marquardt *et al.* 1987), thus suggesting the deleterious effects of chloroquine (and hence the protective effects of bafilomycins) on CD maturation are directly related to their respective effects on lysosome function. Chloroquine-induced inhibition of CD processing from the pre-pro to pro forms (Fig. 5) may be as a result of a negative feedback mechanism aimed to prevent excessive CD synthesis upon inhibition of its maturation in lysosomes, a hypothesis that requires further investigation.

Our previous assessment of chloroquine-induced AV accumulation in cerebellar granule neurons (Shacka *et al.* 2006b) indicated little effect of bafilomycin, leading us to speculate that the cytoprotective effects of bafilomycin A1 were independent of AV accumulation. Our newest evidence however indicates that bafilomycin A1 attenuation of chloroquine-induced death correlates with a significant decrease in AV accumulation and preservation of autophagic flux in differentiated SH-SY5Y cells, differences that may be explained by cell type-specific effects of chloroquine and bafilomycin.

The molecular target for low-dose bafilomycin-mediated neuroprotection remains unresolved, as our cumulative findings predict that it is independent of V-ATPase inhibition (Shacka *et al.* 2006b). One potential target is hypoxia inducible factor-1 α , shown previously to compete with Von Hippel-Lindau tumor suppressor protein for binding to the c subunit of the V0 sector of V-ATPase in a pH-independent manner (Lim *et al.* 2007). Hypoxia inducible factor-1 α is well known to regulate trans-activation of several genes including heme oxygenase-1, an enzyme complex shown recently to regulate both the ALP (Zukor *et al.* 2009) and degradation of WT α -synuclein (Song *et al.* 2009). Thus bafilomycins may regulate neuronal survival through maintenance of the ALP that is in part independent of V-ATPase inhibition.

In summary, we have shown that low-dose bafilomycin protects neuronal cells against both chloroquine and WT α -synuclein-induced cell death in a manner consistent with a potential preservation of the ALP. The ability of bafilomycin to regulate the oligomerization of detergent-insoluble endogenous α -synuclein may be related in part to its ability to preserve CD maturation and decrease the accumulation of detergent insoluble AVs, which may ultimately correspond to an attenuation of neuronal cell death. Further study of plecomacrolide antibiotics such as bafilomycins A1 and B1 is warranted to better understand the role of the ALP in regulating neuron death in neurodegenerative diseases such as PD. Rigorous pharmacokinetics studies are also war-

ranted to determine the relative ability of bafilomycins to cross the blood brain barrier following peripheral administration and reach therapeutic levels in the brain, and if structural analogs can be developed that effectively penetrate the CNS and exhibit a marked decrease in V-ATPase inhibitory activity.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1. Stimulus-induced death of SH-SY5Y cells.

Figure S2. Low-dose bafilomycin attenuates stimulus-induced death of SH-SY5Y cells.

Figure S3. Low-dose bafilomycin attenuates chloroquine-induced cell death of differentiated SH-SY5Y cells.

Figure S4. Inhibition of autophagy induction does not attenuate chloroquine-induced cell death.

Figure S5. Effects of bafilomycin and chloroquine on DA neuron death in *C. elegans*.

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