Bafilomycin A1 Inhibits Chloroquine-Induced Death of Cerebellar Granule Neurons

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ABBREVIATIONS: V-ATPase, vacuolar type ATPase; LC3, microtubule-associated protein light chain-3; CGNs, cerebellar granule neurons
ABSTRACT

Treatment of cells with the macrolide antibiotic bafilomycin A1, an inhibitor of vacuolar (V)-ATPase, or with the lysosomotropic agent chloroquine, has been shown to pharmacologically inhibit autophagy as evidenced by an accumulation of autophagosomes, which in turn causes Bax-dependent apoptosis. However, bafilomycin A1 has also been reported to inhibit chloroquine-induced apoptosis, suggesting a complex interrelationship between these two inhibitors of autophagy. To determine if the cytoprotective effect of bafilomycin A1 on chloroquine-treated cells was dependent on inhibition of V-ATPase, we examined the single and combined effects of bafilomycin and chloroquine on cultured cerebellar granule neurons. When added separately, chloroquine or high concentrations of bafilomycin A1 (≥10 nM) induced a dose-dependent inhibition of autophagy (as measured by an increase in LC3-II, a marker specific for autophagosomes), followed by caspase-3 activation and cell death. When added in combination, bafilomycin A1 potently inhibited chloroquine-induced caspase-3 activity and cell death at concentrations (≤1 nM) that neither altered vacuolar acidification nor inhibited autophagy. The neuroprotective effects of bafilomycin A1 against chloroquine were substantially greater than those produced by Bax deficiency. Bafilomycin A1-induced neuroprotection appeared to be stimulus-specific, as staurosporine-induced death was not attenuated by co-addition of bafilomycin A1. Together these data suggest that in addition to promoting death via inhibition of V-ATPase and autophagy, bafilomycin A1 possesses novel, neuroprotective properties that inhibit Bax-dependent activation of the intrinsic apoptotic pathway resulting from the pharmacological inhibition of autophagy.
INTRODUCTION

Cell death is classified by morphological criteria into distinct categories, including type I apoptotic death and type II autophagic death (Schweichel and Merker, 1973; Clarke, 1990). Apoptosis is triggered by activation of either extrinsic (death receptor-mediated) or intrinsic (mitochondrial-associated) death pathways (Bredesen, 2000; Putcha et al., 2002). Both apoptotic pathways are regulated by pro- and anti-apoptotic members of the Bcl-2 family (Shacka and Roth, 2005) and culminate in the activation of effector caspases, which induce the nuclear condensation, DNA fragmentation and cell shrinkage that define apoptotic morphology (Schweichel and Merker, 1973; Clarke, 1990). Autophagy is a homeostatically regulated pathway whereby autophagosomes fuse with lysosomes for the subsequent degradation and recycling of macronutrients and damaged organelles by lysosomal enzymes (Klionsky and Emr, 2000; Eskelinen, 2005; Lum et al., 2005b). Autophagosomes are defined ultrastructurally as intracellular, double-membraned vesicles encompassing organelles and cytoplasm and biochemically by accumulation of microtubule-associated protein light chain 3-II (LC3-II). LC3 is a microtubule-associated protein that upon processing from its cytoplasmic form (LC3-I) inserts via covalent lipidation into the inner and outer limiting membranes of autophagosomes as LC3-II (Ichimura et al., 2000; Kabeya et al., 2000). Under conditions of limited trophic support, cells induce autophagy as a catabolic means of survival by increasing the turnover of its intracellular components (Lum et al., 2005a). If trophic support remains low, progressive cellular atrophy may lead to autophagic cell death that is defined morphologically by increased numbers of
autophagosomes in a degenerating cell and may be concomitant with apoptotic morphology.

Chloroquine, a widely-prescribed anti-malarial agent, is also indicated for treatment of autoimmune diseases including systemic lupus erythematosus and rheumatoid arthritis (Baird, 2005; Lai et al., 2001; Issa and Ruderman, 2004; Wozniacka and McCauliffe, 2005). As a weak base, chloroquine concentrates in acidic vesicles such as lysosomes and raises their pH, an effect that has been shown to disrupt the function of lysosomal enzymes (de Duve et al., 1974; Seglen and Gordon, 1980). Chloroquine-induced death has been described in many cell types including immature neurons (Zaidi et al., 2001) and HeLa cells (Boya et al., 2003; Boya et al., 2005) and has been shown to be regulated by members of the Bcl-2 family. Previous studies have indicated that chloroquine induces a transient increase in staining of acidic vesicles with LysoTracker® Red, an effect that precedes a disruption in mitochondrial membrane potential and cell death (Boya et al., 2003; Boya et al., 2005). Bafilomycin A1 is a macrolide antibiotic that was characterized initially for its selective inhibition of the vacuolar-type ATPase (V-ATPase) (Bowman et al., 1988; Drose et al., 1993). At nanomolar concentrations, bafilomycin A1 disrupts the vesicular proton gradient and ultimately increases the pH of acidic vesicles (Yoshimori et al., 1991). This disruption of vesicular acidification by both bafilomycin A1 and chloroquine has been proposed to prevent the fusion of autophagosomes with lysosomes (Yamamoto et al., 1998; Boya et al., 2005), resulting in an inhibition of autophagy (Boya et al., 2005). Importantly, this inhibition of autophagy has been shown to precede the disruption of mitochondrial...
membrane potential and Bax/Bak-dependent apoptosis (Boya et al., 2005), thus underscoring an important link between these two death processes.

A recent study has shown that pre-treatment of HeLa cells with a high concentration of bafilomycin A1 (100 nM) inhibits chloroquine-induced apoptosis (Boya et al., 2003). This protective effect of bafilomycin A1 was attributed to its ability to inhibit the initial localization of chloroquine to acidic vesicles, thereby blocking its disruption of both lysosomal function and the subsequent decrease in mitochondrial membrane potential and induction of apoptosis (Boya et al., 2003). Interestingly, this high concentration of bafilomycin A1 is known to inhibit V-ATPase completely (Bowman et al., 1988), induce vacuolar de-acidification (Yoshimori et al., 1991; Boya et al., 2003; Boya et al., 2005) and promote apoptosis (Nishihara et al., 1995; Kinoshita et al., 1996; Kanzawa et al., 2003; Kanzawa et al., 2004; Boya et al., 2005). These previous results led us to investigate whether bafilomycin A1 could also prevent chloroquine-induced death of neuronal cells. In addition, we set out to determine if bafilomycin A1 had any protective effects at concentrations below its reported ability to inhibit vesicular acidification. Results of the present study indicate a dramatic, bafilomycin A1-dependent reduction in chloroquine-induced apoptosis of cerebellar granule neurons (CGNs) by a mechanism that is independent of its effects on vacuolar acidification and is downstream of autophagosome formation.
MATERIALS AND METHODS

Reagents

Unless otherwise noted, reagents were acquired from Sigma (St. Louis, MO).

Animals

C57BL/6J mice were used in all experiments. The generation of mice deficient in bax and caspase-3 has been described previously (Knudson et al., 1995; Kuida et al., 1996; Shindler et al., 1997a). Genetic status was determined by polymerase chain reaction (PCR) analysis of tail DNA extracts as described previously (Kuida et al., 1996; Shindler et al., 1997b). Mice were cared for in accordance with the guidelines of the NIH Guide for the Care and Use of Laboratory Animals. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Cell Culture

CGNs were isolated from postnatal day 7 mice as previously described (Nowoslawski et al., 2005). CGNs were plated in poly-L-lysine coated plates at a density of 1250 cells per mm². 48 well plates were used for viability and caspase activity assays; 100 mm dishes were used for western blot analysis; 4 well Permanox chamber slides (Nalgene) were used for analysis of vacuolar acidification; 4 well glass chamber slides (Nalgene) were used for ultrastructural analysis; and 6 well plates were used for protein degradation assays. CGNs were treated on in vitro day 4. The conditioned media was replaced with fresh media with or without chloroquine (5-40 µM final), in the presence or
absence of bafilomycin A1 (0.1-100 nM final), BOC-aspartyl(Ome)-fluoromethyl ketone (150 µM final; MP Biomedicals, Aurora, OH) or cycloheximide (0.01-1 µg/ml final). Separate cultures of CGNs were also treated for 24h with staurosporine (0.1 µM), in the presence or absence of bafilomycin A1 (1-10 nM final).

**Measurement of Viability and Caspase-3-Like Activity**

Cell viability (via Calcein AM fluorogenic conversion assay; Molecular Probes, Eugene, OR) and caspase-3-like activity (via fluorogenic DEVD cleavage assay) were performed as previously described (Nowoslawski et al., 2005) and were expressed relative to untreated controls.

**Labeling of CGNs with LysoTracker® Red**

Conditioned media was removed from CGNs grown in 4 well, permanox chamber slides. LysoTracker® Red (0.05 µM; Molecular Probes, Eugene, OR) and Calcein AM (2.5 µg/ml; Molecular Probes, Eugene, OR) were prepared in Locke’s Buffer and added to each well for 30 min, 37°C. After this incubation the cells were washed with Locke’s Buffer and cover-slipped. Images were captured using a Carl Zeiss Axioskop microscope equipped with epifluorescence.

**Ultrastructural Analysis of CGNs**

After 24h treatment with chloroquine and/or various concentrations of bafilomycin A1 that began on day 4 *in vitro*, cells were fixed overnight at 4°C with 3% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.45 mM Ca++, rinsed 3 times with
cold buffer at 4°C, and osmicated (2% OsO₄ in PBS, Electron Microscopic Sciences, Fort Washington, PA). Subsequently, wells were rinsed 3 times in deionized H₂O and dehydrated in graded alcohols (30-100%). Cultres were treated overnight with a 1:1 mixture of 100% EtOH and Polybed (Polysciences, Warrington, PA). The 1:1 Polybed/EtOH mixture was replaced with complete Polybed and placed under vacuum for 4h, then replaced by fresh complete Polybed and baked overnight in a 60°C oven under vacuum. Ultrathin sections were prepared with an ultramicrotome, collected onto slot grids, stained with uranyl acetate and lead citrate, and viewed with a JEOL 1200 transmission electron microscope.

Measurement of Degradation of Long-Lived Proteins

Measurement of long-lived protein degradation was performed with minor modifications as previously described (Pattingre et al., 2004). CGNs were radiolabeled on day 3 in vitro with 0.2 µCi/ml of ¹⁴C-L-valine for 24h at 37°C in valine-free Neurobasal media (UCSF Cell Culture Core Facility, San Francisco, CA) and B-27 supplement (Invitrogen). After the radiolabeling period, unincorporated radioisotope was removed by three rinses with PBS (pH 7.4). Cells were then incubated with culture media containing 10 mM valine for 16h at 37°C followed by three rinses with PBS to remove the radioactivity due to the degradation of short-lived proteins. Cells were then treated for 24h at 37°C with chloroquine and/or bafilomycin A1 in media containing 10 mM valine. At this time cells and radiolabeled proteins from the media were precipitated in ice-cold trichloroacetic acid (final concentration =10% vol/vol). The precipitated proteins were separated from the soluble radioactivity by centrifugation at 600 x g for 20 min and
then dissolved in 0.2 N NaOH. Radioactivity was determined by liquid scintillation counting. Protein degradation was calculated by dividing the acid-soluble radioactivity recovered from media by the total radioactivity from the acid-soluble and precipitated fractions of cells and media and was expressed as percent degradation/hour of treatment.

Western Blot

Cells were detached from their substrate by incubation for 5 min at 37°C with Accutase (Innovative Cell Technologies, San Diego, CA). Conditioned media and cells were centrifuged (700 x g, 5 min, 4°C). The supernatant was aspirated and re-suspended with 1 ml ice-cold PBS, then centrifuged again (700 x g, 5 min, 4°C). The resultant pellet was re-suspended in lysis buffer containing 25 mM HEPES, 5 mM EDTA, 5 mM MgCl₂, 1% SDS, 1% Triton X-100, 1 mM PMSF, 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail (Sigma). Cell lysates were sonicated to shear DNA and then centrifuged (10,000 rpm, 10 min, 4°C), and the resultant supernatant (cleared whole cell lysates) was transferred to a fresh tube. Protein levels were determined subsequently via BCA assay (Pierce). Equal amounts of protein were resolved via SDS-PAGE and transferred to PVDF. After transfer blots were cut in half at approximately 37 kDa. The lower molecular weight blots were used initially for the detection of active caspase-3 with an antibody raised against the cleaved or active fragment (Cell Signaling), and the upper molecular weight blots were used for detection of β-III tubulin (Santa Cruz) to normalize for protein loading. Blots were blocked for 1h, RT (5% milk), followed by overnight incubation with primary antibody. Blots were
washed with 1X TBS containing 0.1% Tween 20, then incubated with secondary antibody (goat anti-rabbit IgG, Biorad) for 1h, RT and subsequently washed. Signal was detected using Supersignal chemiluminescence (Pierce). After detection of cleaved caspase-3, the bottom blot was stripped using Restore Western Blot stripping buffer (Pierce) then probed with rabbit anti-LC3 (provided by Uchiyama laboratory), using the same western blot protocol as described above. Blots were scanned for densitometric analysis using Biorad Quantity One® software.

Statistics

Significant effects of treatment were analyzed either by one-factor ANOVA (if three or more groups) or by unpaired, two-tailed t-test (if two groups). Genotype-specific effects of treatment were analyzed for significance via two-factor ANOVA. Post hoc analysis was conducted using Bonferonni’s test. A level of p<0.05 was considered significant.
RESULTS

CGNs were treated with 5-40 µM chloroquine after four days in vitro and their viability and fractional caspase-3-like activity were measured 16-24h later (Figure 1a,b). Chloroquine produced a concentration-dependent decrease in viability (Fig. 1a) and increase in caspase-3-like activity, with significant effects observed at concentrations of chloroquine ≥10 µM. Chloroquine decreased viability to 24% at 20 µM and to 8% at 40 µM (Fig. 1a) while a 6-7 fold increase in caspase-3-like activity was maximal at 20-40 µM (Fig. 1b). For all subsequent experiments, a concentration of 20 µM chloroquine was used. Treatment with the protein synthesis inhibitor cycloheximide (0.1-1 mg/ml) did not alter the magnitude of death induced by chloroquine (data not shown), which suggests that the deleterious effects of chloroquine were not dependent on de novo protein synthesis.

Time course studies were performed to determine the temporal induction of caspase-3-like activity and cell death by chloroquine (Fig. 1c,d). Viability decreased slightly (by 7%) but significantly after 8h and continued to decrease significantly over the next 40h (Fig. 1c). Caspase-3-like activity increased significantly 8h following treatment with chloroquine and was maximal at 16h (6-fold induction), and remained at high levels from 24-48h (Fig. 1d). The temporal pattern of cleaved or “active” caspase-3 was confirmed by western blot analysis (Fig. 2a,b), which was evident at low levels by 8h and was most robust at 16h following treatment with chloroquine.

To determine the effects of chloroquine on autophagosomes, the processing of LC3 was assessed via western blot (Fig. 2a,c-e). Levels of the 18 kDa LC3-I appeared lower at 24-48h following treatment with chloroquine in comparison to control levels at
those times (Fig. 2a,b). LC3-II, the 16 kDa form of LC3 specific for membranes of autophagosomes (Kabeya et al., 2000), was elevated within 4h after chloroquine addition and remained high through 48h (Fig. 2a,c). This increase in LC3-II was also reflected by an increase in the ratio of autophagosome-bound LC3-II to that of cytosolic LC3-I (Fig. 2d).

The effects of bafilomycin A1 on the survival and apoptosis of CGNs were assessed after 24h (Fig. 3). By itself, bafilomycin A1 did not decrease viability or induce caspase-3-like activity at concentrations ≤1 nM, but significantly decreased viability and increased caspase-3-like activity at concentrations ≥10 nM (Fig. 3a,b). Western blot analysis was performed to confirm the effects of bafilomycin A1 on the activity of caspase-3 and to determine its effects on the processing of LC3 (Fig. 4). An increase in cleaved caspase-3 was only apparent upon treatment with 10 nM bafilomycin A1 (Fig. 4a,b). Treatment with bafilomycin A1 at any concentration did not change levels of LC3-I, but 10 nM bafilomycin A1 induced a dramatic increase in levels of LC3-II (Fig. 4a,c), which was also reflected by a dramatic increase in the ratio of LC3-II/LC3-I (Fig. 4d).

The effects of bafilomycin A1 were also assessed in the presence of a toxic dose of chloroquine (Fig. 5). Bafilomycin A1 significantly attenuated the chloroquine-induced decrease in viability at all concentrations tested, and was maximal at 1 nM (viability of 88% with bafilomycin A1 versus 24% without; Fig. 5a). Although concentrations of 10-100 nM bafilomycin A1 by themselves decreased viability (Fig. 3a), they significantly attenuated the still larger decrease in viability induced by chloroquine (Fig. 5a). The chloroquine-induced increase in caspase-3-like activity was reduced only upon the co-
addition of 0.3-1 nM bafilomycin A1, and was maximal at 1 nM (3-fold reduction; Fig. 5b).

To determine whether bafilomycin A1 affected the toxicity of a classical apoptosis-inducing stimulus, CGNs were treated for 24h with 0.1µM staurosporine (Fig. 5c), a concentration of this broad spectrum kinase inhibitor that significantly increases caspase-3-like activity and apoptosis in our culture system (data not shown). Treatment with staurosporine for 24h significantly reduced viability to an average of 44%, an effect that was not altered by co-administration with 1 nM bafilomycin A1. Treatment with 10 nM bafilomycin A1, rather than protecting CGNs from staurosporine-induced death, further decreased the viability of staurosporine-treated CGNs to 20%.

Western blot analysis was performed to confirm the effects of bafilomycin A1 on the activity of caspase-3 and to determine its effects on the processing of LC3, in the presence of chloroquine (Fig. 6). The chloroquine-induced increase in cleaved caspase-3 was attenuated by co-treatment with ≤1 nM bafilomycin A1 (Fig. 6a,b). Bafilomycin A1 did not attenuate chloroquine-induced levels of LC3-II but appeared to increase levels of LC3-I, which is reflected by a reduction in the ratio of LC3-II to LC3-I at all concentrations of bafilomycin A1 tested (Fig. 6a,c).

Vacuolar acidification was measured following 24h treatment with chloroquine and/or bafilomycin A1 by incubation with LysoTracker® Red, in the presence of the viability marker calcein AM (Fig. 7). By itself, 1 nM bafilomycin A1 did not alter the staining pattern of LysoTracker® Red nor did it reduce viability in comparison to control cells (Fig. 7a,b). In contrast, treatment with 10 nM bafilomycin A1 not only prevented any detection of vacuolar acidification by LysoTracker® Red but also decreased
numbers of viable CGNs (Fig. 7c), an effect that was similar in the presence or absence of chloroquine (Fig. 7c,f). Treatment with chloroquine decreased numbers of viable CGNs (Fig. 7d). However, the population of CGNs that survived 24h treatment with chloroquine exhibited a greater intensity of LysoTracker® Red staining than that observed in control cells (Fig. 7d). The co-addition of 1 nM bafilomycin A1 and chloroquine (Fig. 7e) increased numbers of viable neurons that also exhibited a concomitant increase in LysoTracker® Red staining intensity, in comparison to treatment with chloroquine or 1 nM bafilomycin A1 alone.

Electron microscopy was performed on CGNs to determine the effects of 24h treatment with chloroquine with or without bafilomycin A1 on ultrastructural morphology. There were no morphological differences between control CGNs (Fig. 8a) and cells treated with 0.3 nM bafilomycin A1 (Fig. 8b). Treatment with 10 nM bafilomycin A1 (Fig. 8c) induced the accumulation of large, swollen, single-membraned vacuoles in the cytoplasm with electron-dense deposits, in addition to apoptotic nuclei. Chloroquine (Fig. 8d, 8g) induced the formation of apoptotic nuclei and also the accumulation of single membraned vacuoles with dense, electron dense deposits and a whorled appearance, possibly indicating mature, degradative autophagosomes or autophagolysosomes (Eskelinen, 2005). The vacuoles that accumulated upon treatment with 10 nM bafilomycin A1 (Fig. 8c) were much larger and swollen than those that accumulated upon treatment with chloroquine and were not whorled in appearance, which may indicate a population of mature autophagosomes whose fusion with lysosomes was blocked, as suggested previously (Yamamoto et al., 1998). Co-treatment with chloroquine and 0.3 nM bafilomycin A1 (Fig. 8e, 8h) attenuated the
chloroquine-induced appearance of apoptotic nuclei but did not prevent the accumulation of single membranated, whorled vacuoles with electron-dense deposits. Co-treatment with chloroquine and 10 nM bafilomycin A1 (Fig. 8f, 8i) induced the accumulation of large, swollen, single membranated vacuoles in addition to apoptotic nuclei.

Treatment with chloroquine has been shown previously to inhibit macroautophagy by inhibiting the degradation of long-lived proteins (Boya et al., 2005). In the present study, degradation of long-lived proteins was measured after 24h treatment with chloroquine and/or bafilomycin A1 to determine if chloroquine had similar effects on macroautophagy in CGNs, and if bafilomycin A1 differentially affected macroautophagy in the presence or absence of chloroquine (Fig. 9). Chloroquine significantly inhibited the degradation of long-lived proteins versus control cultures, an effect that was not altered by co-treatment with either 1 or 10 nM bafilomycin A1. One nM bafilomycin A1 by itself did not alter the degradation of long-lived proteins versus control. Although 10 nM bafilomycin A1 appeared to decrease the degradation of long-lived proteins in comparison to control, this effect did not reach statistical significance.

Cultures were prepared from Bax-deficient mice to determine the role of this pro-apoptotic member of the Bcl-2 family in chloroquine-induced death (Fig. 10). The targeted deletion of Bax significantly attenuated the chloroquine-induced decrease in viability (Fig. 10a) and increase in caspase-3-like activity (Fig. 10b), measured at 16-24h, but did not provide any greater protection than that achieved with 1 nM bafilomycin A1 (Fig. 10a,b). Conversely, Bax deficiency prevented the reduction in viability and increase in caspase-3-like activity that was induced by treatment for 24h with 10 nM
bafilomycin A1 (Fig. 10c,d). The effects of targeted deletion of Bax on the activity of caspase-3 were confirmed via western blot (Fig. 10e). Furthermore, western blot analysis indicated that Bax deficiency did not prevent the chloroquine-induced increase in LC3-II (Fig. 10e).

Since chloroquine induced a dramatic increase in the activity of caspase-3, cultures of CGNs were prepared from caspase-3-deficient mice to determine the relative positioning of this effector caspase in the pathway of cell death induced by chloroquine. The absence of caspase-3 did not prevent the chloroquine-induced decrease in viability (Fig 11a), although a significant attenuation in caspase-3-like activity was observed (Fig. 11b). To determine if other caspases, e.g. caspase-9, may be critical for chloroquine-induced death, CGNs were treated with BOC-aspartyl(Ome)-fluoromethyl ketone, a broad-spectrum caspase inhibitor. Treatment with this inhibitor did not attenuate chloroquine-induced death (Fig. 11c), but did significantly attenuate the chloroquine-induced increase in caspase-3-like activity (Fig. 11d).
DISCUSSION

Inhibition of autophagy by chloroquine or bafilomycin A1 occurs via a disruption in the pH of acidic vesicles, which prevents the fusion of autophagosomes with lysosomes and thus increases the cellular burden of autophagosomes, and has been shown to precede and possibly induce Bax- and mitochondrial-dependent apoptosis (Zaidi et al., 2001; Boya et al., 2003; Boya et al., 2005). In the present study, chloroquine produced a concentration- and time-dependent increase in caspase-3-like enzymatic activity and decrease in the viability of CGNs (Figs. 1-2). The chloroquine-induced death of CGNs was due to the engagement of the intrinsic apoptotic pathway, as evidenced by a Bax-dependent increase in caspase-3 activity and decrease in cell viability (Fig 10). Chloroquine also induced a dramatic increase in LC3-II (Figs. 2, 6) that preceded activation of caspase-3 (Fig. 2) and was Bax-independent (Fig. 10). In addition, treatment with ≥10 nM bafilomycin A1 increased levels of LC3-II (Fig. 4) and caused an accumulation of vacuoles (Fig. 8), enhanced Bax-dependent apoptosis (Figs. 3-4,10) and severely impaired vacuolar acidification (Fig. 7), results that are similar to those of previous studies (Boya et al., 2003; Boya et al., 2005). Importantly, results of the present study confirm and extend previous studies suggesting that an alteration in autophagy precedes and initiates apoptotic cell death (Boya et al., 2005; Boya et al., 2003; Lee and Baehrecke, 2001; Martin and Baehrecke, 2004; Gonzalez-Polo et al., 2005).

Co-treatment with bafilomycin A1 dramatically and significantly attenuated chloroquine-induced apoptosis (Figs. 5-6), an effect that was characterized by an inverted, U-shaped concentration-dependence. This effect was maximal at a
concentration of bafilomycin A1 (0.3-1 nM) that by itself neither altered vacuolar acidification (Fig. 7) nor induced the formation of autophagosomes (Fig. 4). This concentration of bafilomycin A1 was shown previously to have minimal inhibitory effect on V-ATPase in a cell-free system (Bowman et al., 1988; Drose et al., 1993). However, 10-100 nM bafilomycin A1 still inhibited chloroquine-induced death, though not as robustly as with lower concentrations, which is due likely to bafilomycin A1-dependent induction of the intrinsic apoptotic pathway at ≥10 nM. It is possible that high concentrations of bafilomycin A1 prevent a distinct, caspase-independent pathway of chloroquine-induced cell death, since chloroquine-induced caspase-3-like activity was not affected by ≥10 nM bafilomycin A1. Nevertheless, 100 nM bafilomycin A1 has been shown previously to inhibit chloroquine-induced apoptosis of HeLa cells (Boya et al., 2003), which suggests cell-type-specific effects of bafilomycin A1 and chloroquine. Effects of bafilomycin A1 in various cell culture models have been typically reported using concentrations ≥10 nM (Boya et al., 2003; Boya et al., 2005; Kanzawa et al., 2003; Kanzawa et al., 2004). Together, our results reveal a novel, concentration-dependent dichotomy of bafilomycin A1 function such that ≤ 1 nM bafilomycin A1 inhibits chloroquine-induced apoptosis apart from its inhibition of vacuolar acidification. In addition, the protective effects of bafilomycin A1 may be limited to stimuli that induce alterations in autophagy, since it did not prevent death caused by staurosporine, a protein kinase inhibitor and classical apoptosis-inducing agent (Fig. 5).

In the present study, CGNs that survived the 24h chloroquine incubation stained intensely for LysoTracker® Red. A chloroquine-induced increase in LysoTracker® Red staining intensity has been documented previously in other cell types (Boya et al.,
and occurs concomitantly with an increase in autophagosome number, yet dissipates only in cells exhibiting a subsequent disruption in mitochondrial membrane potential and resultant apoptosis (Boya et al., 2005). Thus the CGNs staining intensely with LysoTracker® Red likely represent the viable population of neurons that have intact mitochondrial function, although mitochondrial function was not directly measured in the present study. Although LysoTracker® Red does not discriminate between different types of acidic vesicles or organelles, its staining pattern may represent a population of late or mature autophagosomes, as suggested by ultrastructural analysis (Fig. 8), that become increasingly acidic during their maturation (Dunn, Jr., 1990; Punnonen et al., 1992). By disrupting the function of lysosomes, chloroquine may prevent their fusion with late autophagosomes, resulting ultimately in the accumulation of late autophagosomes.

One nM bafilomycin A1 not only increased the population of CGNs that survived the chloroquine insult but also increased numbers of viable CGNs with intensely-stained, LysoTracker® Red-positive acidic vesicles (Fig. 7). A relative lack of apoptotic nuclei was seen ultrastructurally after treatment for 24h with chloroquine plus 0.3 nM bafilomycin A1 (Fig. 8), confirming the results of caspase activity assays (Fig. 5) and western blot analysis (Fig. 6). In addition, ultrastructural analysis suggests that there is little if any effect of 0.3 nM bafilomycin A1 on the chloroquine-induced accumulation of autophagosomes. Together, these data suggest that bafilomycin A1, at concentrations below its ability to inhibit V-ATPase, may prevent not only the de-stabilization of acidic vesicles (as evidenced by even greater numbers of viable cells exhibiting intense
LysoTracker® Red staining) but also the disruption of mitochondrial function that would lead to induction of the intrinsic apoptotic pathway.

Conversely, treatment with 10 nM bafilomycin A1 completely prevented LysoTracker® Red staining, both in the presence or absence of chloroquine. This may suggest that bafilomycin A1 (via inhibition of V-ATPase) prevents the fusion and maturation of early autophagosomes, as described previously (Yamamoto et al., 1998), thus accumulating a population of autophagosomes with a pH too high for detection with LysoTracker® Red. Ultrastructural analysis indeed confirmed the accumulation of single membraned, cytoplasmic vacuoles in CGNs treated with 10 nM bafilomycin A1 that appeared much larger and swollen compared to those in cells treated with chloroquine or chloroquine plus 0.3 nM bafilomycin A1 (Fig. 8) and were not whorled in appearance, which may indicate a population of mature autophagosomes whose fusion with lysosomes with lysosomes was prevented. LC3-II has been documented in both early and late autophagosomes (Jager et al., 2004), which would indicate why both chloroquine and 10 nM bafilomycin A1 increased levels of LC3-II yet produced differential staining patterns with LysoTracker® Red and differential vacuolar morphology.

Bafilomycin A1 attenuated the chloroquine-induced increase in the ratio of LC3-II/LC3-I (Fig. 6). However, these changes were due primarily to increased levels of cytosolic LC3-I with little change in levels of LC3-II (Fig. 6). Results of ultrastructural analyses (Fig. 8) do not suggest a decreased accumulation in autophagosomes upon co-treatment with chloroquine and bafilomycin A1. A possible interpretation of these results would be that bafilomycin A1, especially at low concentrations (≤ 1 nM) either
induces autophagy as a survival response to chloroquine or attenuates the chloroquine-induced inhibition of autophagy. An increase in levels of LC3-II has been reported from both the induction and inhibition of autophagy (Tanida et al., 2005), which could explain why ≤ 1 nM bafilomycin A1 did not decrease levels of LC3-II. However, neither 1 nor 10 nM bafilomycin A1 prevented the chloroquine-induced inhibition of autophagy (Fig. 9), which suggests that bafilomycin A1 does not prevent chloroquine-induced death by altering autophagosome formation and/or recycling.

Although our results clearly indicate that chloroquine-induced death is in part Bax-dependent, the degree of protection afforded by 1 nM bafilomycin A1 against chloroquine was substantially greater than that provided by Bax deficiency. Considering that bafilomycin A1 significantly attenuates activation of caspase-3, we propose that the putative protective mechanism of bafilomycin A1 lies upstream of Bax and may prevent activation of the intrinsic apoptotic pathway (Fig. 12). Since Bax deficiency virtually prevented the chloroquine-induced activation of caspase-3 (Fig. 10), this additional protective effect of bafilomycin A1 cannot be attributed to its functional inhibition of molecules such as Bak, which shares redundant regulatory functions with Bax (Wei et al., 2001; Putcha et al., 2002). In contrast, Bax deficiency significantly attenuated the induction of caspase-3-like activity and the reduction in viability resulting from 10 nM bafilomycin A1 (Fig. 10), which suggests that similar to the effects of chloroquine, the inhibition of autophagy by high concentrations of bafilomycin A1 induces Bax-dependent apoptosis.

Although chloroquine induced a robust activation of caspase-3 (Figs. 1-2, 5-6, 10), the targeted deletion of caspase-3 and broad pharmacological inhibition of
Caspases did not prevent chloroquine-induced death (Fig. 11). Similar results were obtained previously from our laboratory with cultured telencephalic neurons (Zaidi et al., 2001), which suggests that the commitment point for chloroquine-induced neuron death lies upstream of caspase activation. These findings are in contrast to the chloroquine-induced death of HeLa cells, which was attenuated upon treatment with a general caspase inhibitor (Boya et al., 2003). Activation of the intrinsic apoptotic pathway in chloroquine-induced neuron death is clear, considering that the temporal and concentration-dependent, chloroquine-induced decrease in viability closely follows the pattern of caspase-3 activity and that 1 nM bafilomycin A1 dramatically attenuates activation of caspase-3 concomitant with decreased cell death. This conclusion is further supported by the observed decrease in caspase-3 activity and cell death in the absence of Bax. Together, the results of this study suggest that the commitment point of chloroquine-induced neuron death lies upstream of caspase activation (Fig. 12) at the level of Bax, and that the protective effects of bafilomycin A1 lie upstream of Bax.

Chloroquine-induced neurotoxicity has been documented in humans (James, 1988; Phillips-Howard and ter Kuile, 1995; Telgt et al., 2005) and has been linked to cerebellar ataxia (James, 1988). Animal models of chloroquine treatment have indicated the cerebellar accumulation of lipoprotein (Ivy et al., 1989; Fischer and Nelson, 1974) which suggests that chloroquine is an in vivo inhibitor of neuronal autophagy. Studies assessing the in vivo effects of bafilomycins are limited in number (Myers et al., 2001; Hettiarachchi et al., 2004) and the ability of bafilomycins to cross the blood brain barrier is unknown, thus warranting future pharmacokinetic analysis. In vitro analysis of CGNs has shown a bafilomycin A1-induced inhibition of glutamate release (Cousin et
al., 1995), and bafilomycin A1 reportedly inhibits the vesicular uptake of many neurotransmitters including but not limited to glutamate, serotonin and GABA (Moriyama and Futai, 1990; Roseth et al., 1995). It is unlikely that the protective effects of bafilomycin A1 are due to alterations in neurotransmitter uptake/release, since these actions are observed at micromolar concentrations and 1 nM bafilomycin A1, its most effective neuroprotective concentration, does not inhibit glutamate uptake (Roseth et al., 1995). Finally it is worth noting that lysosomal dysfunction has been implicated in a variety of neurodegenerative conditions including Alzheimer’s, Parkinson’s, and Huntington’s diseases (Bahr and Bendiske, 2002). Whether bafilomycin A1 has neuroprotective action in any of these conditions requires further investigation.
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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Chloroquine decreases cell viability and increases caspase-3-like activity. Viability (a) expressed as % control (CTL) and fractional caspase-3-like activity (b) measured 24h following treatment with chloroquine (5-40 µM). Viability (c) and caspase-3-like activity (d) were also measured for up to 48h following treatment with 20 µM chloroquine. Three independent experiments of at least three replicates represent each time point and concentration tested. Significant effects of treatment and time were assessed via one-factor ANOVA with Bonferroni’s post hoc test. For a & b, p<0.05 (a compared to CTL; b compared to 5 µM chloroquine; c compared to 10 µM chloroquine; d compared to 20 µM chloroquine). For c & d, p<0.05 (a compared to 0h; b compared to 8h; c compared to 16h; d compared to 24h).

Fig. 2. Chloroquine induces biochemical markers of apoptosis and autophagy. Representative western blot (a) measures cleaved caspase-3 and processing of LC3 in non-treated (NT) samples at 0h and in control (CTL) or chloroquine (20 µM; CQ)-treated lysates from 4-48h. At least three independent experiments were used to assess levels cleaved caspase-3 (b), LC3-I (c) and LC3-II (d), expressed relative to levels of β-III-tubulin, and the ratio of LC3-II/I (e).

Fig. 3. Bafilomycin A1 (BafA1) concentration-dependently decreases viability and increases caspase-3-like activity. Viability (a) and fractional caspase-3-like activity (b) were measured 24h following the addition of BafA1 (0.1-100 nM). Three independent experiments of at least three replicates represent each time point and concentration.
tested. Significant effects of treatment were assessed via one-factor ANOVA with Bonferonni’s post hoc test; p<0.05 (*compared to 0, 0.1 and 0.3 nM BafA1; †compared to 10 nM BafA1).

Fig. 4. Bafilomycin A1 (BafA1) concentration-dependently increases markers of apoptosis and autophagy. Representative western blot (a) measures cleaved caspase-3 and processing of LC3 in lysates following 24h addition of BafA1. At least three independent experiments were used to assess levels of cleaved caspase-3 (b), LC3-I and LC3-II (c) expressed relative to levels of β-III-tubulin, and the ratio of LC3-II/LC3-I (d).

Fig. 5. Bafilomycin A1 (BafA1) concentration-dependently attenuates chloroquine-induced decrease in viability and increase in caspase-3-like activity. Viability (a) and fractional caspase-3-like activity (b) were measured 24h following the co-addition of 20 μM chloroquine (CQ) and BafA1. BafA1 did not prevent the decrease in viability following 24h treatment with 0.1 μM STS (c). Three independent experiments of at least three replicates represent each concentration tested. For a&b, significant effects of BafA1 (in the presence or absence of CQ) were assessed via one-factor ANOVA with Bonferonni’s post hoc test; p<0.05 (a compared to CTL; b compared to CQ; c compared to 0.1 nM BafA1; d compared to 0.3 nM BafA1; e compared to 1 nM BafA1). For c, significant effects of staurosporine (in the presence or absence of BafA1) were assessed via two-factor ANOVA and concentration-dependent differences of BafA1 were assessed via one-factor ANOVA with Bonferonni’s post hoc test; p<0.05.
Fig. 6. Bafilomycin A1 (BafA1) dose-dependently attenuates chloroquine-induced cleavage of caspase-3 and the ratio of LC3-II/LC3-I. Representative western blot (a) indicates cleaved caspase-3 and processing of LC3 in samples treated for 24h with BafA1 (0.3-10 nM) in the presence or absence of 20 µM chloroquine (CQ). At least three independent experiments were used to assess levels of cleaved caspase-3 (b), LC3-I and LC3-II (c) expressed relative to levels of β-III-tubulin, and the ratio of LC3-II to LC3-I (d).

Fig. 7. Effects of chloroquine (CQ) and bafilomycin A1 (BafA1) on the labeling of acidic vesicles. CGNs were treated for 24h as follows: (a) CTL; (b) 1 nM BafA1; (c) 10 nM BafA1; (d) 20 µM CQ; (e) CQ+1 nM BafA1; (f) CQ+10 nM BafA1; and were subsequently labeled for acidic vesicles using Lysotracker Red and for viability using Calcein AM. Each experiment was repeated three times with similar results. Scale bar = 100 microns.

Fig. 8. Effects of chloroquine (CQ) and bafilomycin A1 (BafA1) on morphology of CGNs as assessed ultrastructurally by electron microscopy (EM). CGNs were treated for 24h as follows: (a) CTL; (b) 0.3 nM BafA1; (c) 10 nM BafA1; (d) 20 µM CQ; (e) CQ+0.3 nM BafA1; (f) CQ+10 nM BafA1; and were subsequently processed for EM. Boxes in d-f refer to enlarged images of vacuoles represented in g-i respectively. Arrows point to apoptotic nuclei. Arrowheads point to vacuoles with electron dense granules and
whorled appearance. Asterisks indicate large, swollen vacuoles with electron dense granules. Scale bar =2 microns for a-f and 0.5 microns for g-i.

**Fig. 9.** Bafilomycin A1 (BafA1) does not attenuate chloroquine (CQ)-induced inhibition of macroautophagy. CGNs radiolabeled with $^{14}$C valine were treated with CQ, BafA1 or CQ+BafA1 for 24h and processed subsequently for degradation of long-lived proteins. Data represent mean±S.E.M. of three independent experiments and are expressed as percent degradation /hour of treatment. Significant effects of treatment were assessed via one-factor ANOVA with Bonferonni’s post hoc test; p<0.05 (*compared to CTL-treated cultures).

**Fig. 10.** The cytoprotective effects of bafilomycin A1 (BafA1) are Bax-independent. Control (CTL)- and chloroquine (CQ; 20 µM)-treated, wild type (WT) and Bax-deficient (KO) CGNs were assessed for viability (a&c) and fractional caspase-3-like activity (b&d) in the presence of 1 nM (a&b) or 10 nM (c&d) BafA1. At least three independent experiments of at least three replicates represent each concentration and genotype tested. Representative western blot (e) indicates cleaved caspase-3 and processing of LC3 in WT and KO lysates following 24h treatment with CTL or CQ. NT=no treatment, 0h. For a&b, significant effects of genotype versus treatment were assessed via two-factor ANOVA and Bonferonni’s post hoc test and significant treatment effects were assessed via one-factor ANOVA and Bonferonni’s post hoc test; p<0.05 (acompared to respective WT treatment pair; bcompared to genotype-matched CTL; ccompared to genotype-matched treatment with 1 nM BafA1; dcompared to genotype-matched treatment with CQ). For c&d, significant effects of genotype versus treatment were
assessed via two-factor ANOVA and Bonferonni’s post hoc test and significant
treatment effects were assessed via unpaired, two-tailed t-test; p<0.05 (a compared to respective WT treatment pair; †compared to genotype-matched CTL).

Fig. 11. Neither caspase-3 deficiency nor pharmacological inhibition of caspases prevents chloroquine-induced death. Cell viability (a&c) and fractional caspase-3-like activity (b&d) were measured 24h following treatment with chloroquine (CQ) or control (CTL) in wild type (WT) and caspase-3-deficient (KO) CGNs (a&b) or in CGNs co-treated with the broad spectrum caspase inhibitor BOC-aspartyl(Ome)-fluoromethyl ketone (BAF, 150 µM; c&d). At least three independent experiments of at least three replicates represent each concentration and genotype tested. For a&b, significant effects of genotype were assessed via two-tailed, unpaired t test; p<0.05 (*compared to CTL). For c&d, significant effects of treatment were assessed via one-factor ANOVA and Bonferonni’s post hoc test; p<0.05 (*compared to CTL; †compared to CQ).

Fig. 12. Proposed model indicates concentration-dependent effects of bafilomycin A1 (BafA1) on chloroquine-induced apoptosis. At low concentrations, BafA1 inhibits chloroquine-induced apoptosis, either upstream of or at the level of Bax. High concentrations of BafA1 do not prevent chloroquine-dependent apoptosis, and in the absence of chloroquine, appear to inhibit autophagy and induce Bax-dependent apoptosis.
Fig 1

a) Viability (% CTL) vs. μM Chloroquine

b) Fract. Casp.-3-Like Activ. vs. μM Chloroquine

c) Viability (% CTL) vs. Time (h)

d) Fract. Casp.-3-Like Activ. vs. Time (h)
**Fig 2**

(a) Western blot analysis showing the expression levels of CL Caspase-3, LC3-I, LC3-II, and Tubulin under different conditions. The blots are labeled with NT, CTL, CQ, CTL+CQ, and CTL+CQ groups at various time points (0h, 4h, 8h, 16h, 24h, 48h).

(b) Graph showing the time course of Cleaved Caspase-3/Tubulin ratio for CTL and CQ groups.

(c) Graph showing the time course of LC3-I/Tubulin ratio for CTL and CQ groups.

(d) Graph showing the time course of LC3-II/Tubulin ratio for CTL and CQ groups.

(e) Graph showing the time course of LC3-II/LC3-I ratio for CTL and CQ groups.
Fig 5
Fig 6
Fig 9
Fig 10
Chloroquine
BafA1 (≥10 nM)

Inhibition of Autophagy

Bax-dependent Induction of Intrinsic Apoptotic Pathway

Commitment Point for Death

Activation of Caspase-3

Fig 12