The Effects of a Mitochondrial Targeted Peptide (Elamipretide/SS31) on BAX Recruitment and Activation During Apoptosis

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Research note

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Abstract

Objective: Elamipretide (SS31) is a mitochondria-targeted peptide that has reported functions of stabilizing mitochondrial cristae structure and improving mitochondrial bioenergetics. Several studies have documented cell protective features of this peptide, including impairment of intrinsic apoptosis by inhibiting the recruitment and activation of the pro-apoptotic BAX protein. We used live-cell imaging of ARPE-19 cells expressing fluorescently labeled BAX, cytochrome c, and a mitochondrial marker to investigate the effect of elamipretide on the kinetics of BAX recruitment, mitochondrial outer membrane permeabilization (as a function of cytochrome c release), and mitochondrial fragmentation, respectively.

Result: In nucleofected and plated ARPE-19 cells, elamipretide accelerated the formation of larger mitochondria. In the presence of the apoptotic stimulator, staurosporine, cells treated with elamipretide exhibited moderately slower rates of BAX recruitment. Peptide treatment, however, did not significantly delay the onset of BAX recruitment or the final total amount of BAX that was recruited. Additionally, elamipretide showed no impairment or delay of cytochrome c release or mitochondrial fragmentation, two events associated with normal BAX activation during cell death. These results indicate that the protective effect of elamipretide is not at the level of BAX activity to induce pro-apoptotic mitochondrial dysfunction after the initiation of staurosporine-induced apoptosis.

Introduction

Intrinsic apoptosis is tightly regulated by mitochondrial dysfunction that is controlled by proteins of the BCL2 gene family [1], which are grouped on the basis of sharing BCL2 Homology (BH) domains. In healthy cells, the pro-apoptotic members of this family (principally BAX and BAK) function as participants in mitochondrial fusion [2–4]. BAX is a latent protein that predominantly resides in a globular conformation in the cytosol, but also in a state of equilibrium with the mitochondrial outer membrane (MOM). Anti-apoptotic BCL2 family proteins, such as BCL-X, function to shuttle BAX back into the cytosol under steady state conditions [5]. During the activation of cell death, cells up-regulate or activate smaller pro-apoptotic proteins that contain only the third BH domain (BH3-only proteins) that function to inhibit anti-apoptotic proteins and facilitate a conformation change in BAX and BAK, allowing them to interact and embed in the MOM and form dimers. Activated BAX can then enable self-activation with additional cytosolic BAX, resulting in the further recruitment of BAX to the MOM and the formation of large molecular weight oligomers. Kinetic studies show that the recruitment phase follows a sigmoidal function [6] and is completed in a period of approximately 20–30 minutes.

A principal function of BAX activation is the permeabilization of the MOM (MOMP) to allow the release of a variety of pro-apoptotic signaling molecules such as cytochrome c and SMAC/Diablo. Live cell imaging studies in tissue culture cells undergoing apoptosis show that MOMP and the release of cytochrome c occurs nearly instantaneously with the initiation of BAX recruitment and well before the substantive formation of large oligomers [6, 7]. The continued accumulation of BAX to the MOM appears to coincide with larger pore formation [6, 8, 9] that may allow for evulsions of the mitochondrial inner membrane and

the release of mitochondrial DNA [10]. At the end of BAX recruitment mitochondria undergo dramatic fragmentation [11]. This process is significantly reduced in cells with impaired DRP1 function [12], but also in the presence of mutant BAX which has normal fusion activity and impaired function during apoptosis [11], suggesting that these proteins co-operate in a fission complex. Consistent with this, BAX aggregates typically co-localize with DRP1 and associate with mitochondrial scission sites in apoptotic cells [13]. While not completely understood, the relationship between DRP1 and BAX centers around regulation of cardiolipin transport and cristae structure. In healthy cells, cardiolipins are predominantly localized to the mitochondrial inner membrane (MIM), where they help stabilize cristae. During apoptosis, cardiolipins are transferred from the inner membrane to the MOM. This, among other events, has the effect of destabilizing the cristae structure and promoting increased curvature of the MOM. Evidence suggests that DRP1 regulates the process of cristae destabilization [14]. Cristae destabilization also frees cytochrome c into the intermembrane space. BAX accumulation is favored at the sites of high cardiolipin density and increased membrane curvature, prompting some interpretations that DRP1 is required to stimulate BAX recruitment to these regions [15]. Loss of DRP1, however, does not prevent the initiation and early recruitment of BAX [11], nor does it block MOMP and the release of SMAC/Diablo [16, 17], although cytochrome c release is impaired because it has not been released from the cristae [14, 17]. Interestingly, in cells with absent or impaired DRP1 function, BAX recruitment is limited to approximately 75% of the level found in normal cells.

The mitochondrial-targeted peptide elamipretide (SS31) has consistently been shown to ameliorate age-related conditions affecting muscle, cardiac, ocular, and nephrotic tissues [18–22] and has been shown to be protective in animal models of neurodegeneration [23–27]. A variety of factors could account for the beneficial effects of elamipretide including stabilization of mitochondrial cristae structure [22], modulation of lipid surface properties including charge [28], and improved mitochondrial function and transport [27]. Some reports suggest that elamipretide also impairs the recruitment of BAX to the mitochondria [24] and prevents mitochondrial fission [21] during apoptosis, which is consistent with a role in attenuating cell death. Given the ability of elamipretide to associate with cardiolipins and to stabilize cristae structure [22], we hypothesized that elamipretide may alter BAX recruitment to the MOM and/or inhibit downstream events associated with BAX activation such as MOMP and mitochondrial fragmentation. In this report, we document the effect of the mitochondria-targeted peptide in modulating BAX recruitment kinetics using live cell imaging of fluorescently tagged BAX fusion proteins in tissue culture cells undergoing staurosporine (STS)-induced apoptosis, with the expectation that elamipretide would mimic the effects of impaired DRP1 function. These studies show that elamipretide, while accelerating the formation of larger mitochondria in cells, has the ability to moderately slow the rate of BAX recruitment, but does not prevent either MOMP, including the release of cytochrome c, or mitochondrial fragmentation. These studies indicate that the protective effect of elamipretide is not directly as a consequence of modulating the activation of BAX or preventing BAX-mediated mitochondrial dysfunction.

Methods

Cell culture and nucleofection

ARPE-19 cells (immortalized human adherent retinal pigmented epithelial cells) were a gift from Dr. Aparna Lakkaraju (University of California-San Francisco, formerly University of Wisconsin-Madison) and were used in live-cell imaging experiments until passage 25. These cells maintain a relatively stable profile in the Z plane during growth, which improves the ability to track organelles during live-cell imaging experiments. Cells were cultured in DMEM:F-12 (Hyclone, GE Healthcare Life Sciences, Marlborough, MA) supplemented with 10% FBS (Atlanta Biologicals, Norcross, GA) and 1% penicillin streptomycin (Cellgro Mediatech, Inc., Manassas, VA) at 37°C and 5% CO₂.

Plasmids used for these experiments were obtained as follows. MitoBFP and mCherry-BAX were cloned in the laboratory using vectors TagBFP-N (Evrogen, Moscow, Russia) and pmCherry-C1 (Clontech, Mountainview, CA) as described previously [6]. Cytochrome c-GFP (Addgene plasmid #41182) and SMAC-GFP (Addgene plasmid #40881) were gift from Douglas Green's laboratory. All transgenes were run using the CMV immediate early promoter. For nucleofection, 1 million cells were combined with 3–5 µg of each plasmid and nucleofected using protocol DN-100 in an Amaxa Nucleofector (Lonza, Basel, Switzerland). After nucleofection $3.5\text{--}5.0 \times 10^5$ cells were plated on 35 mm glass bottomed (No. 1.5 optical grade) MatTek dishes (Ashland, MA) and allowed to recover for 16–24 hrs. Cells were then incubated with different concentrations of elamipretide (in DMSO) or DMSO, and apoptosis was induced by the addition of STS to a final concentration of 1 µM. Cells were maintained in DMEM:F12 media until ready for imaging.

Live-cell imaging and analysis

For imaging sessions, media was removed and replaced with recording media (10 µM HEPES containing 1.26 µM CaCl₂, 0.49 µM MgCl₂, 0.4 µM MgSO₄, 5.33 µM KCl, 0.44 µM H₂PO₄, 4.2 µM NaHCO₃, 13.8 µM NaCl, 0.34 µM HPO₄ and 5 g/L glucose). Live cell imaging was performed on an Andor Revolution XD spinning disc confocal microscope (Andor, Belfast, Northern Ireland) comprised of the Nikon Eclipse Ti inverted microscope, Nikon objectives, the iXon x3 897 EM-CCD camera, a Yokogawa CSU-X1 confocal spinning disk head, the Andor Laser Combiner with four solid state lasers, an ASI motorized stage with Piezo-Z, and an Okolab CO₂ cage incubator for temperature and CO₂ control at 37°C and 5% CO₂ (CO₂ was kept off during the experiments). Using the time-lapse function, ARPE-19 images were taken every 2 minutes beginning 14–17 hours post STS addition. All images were taken a 100X oil emersion objective (numerical aperture = 1.49) and consisted of 20–30 optical sections of 0.22 µm (Nyquist steps). All imaging was performed under the same laser intensity (20%), electron-multiplying gain, and exposure time (200 ms).

Image analysis was conducted using IMARIS 9.2.1 software (Neuroscience package, Bitplane Inc., South Windsor, CT) as described previously [6, 11]. Data used for analysis was subject to automatic background subtraction and Gaussian filtering but were not processed through a deconvolution step. Previously, we showed that while deconvolution may affect mitochondrial volume by an increase in the point spread

function in the Z-plane, the 3-dimensional data collected from tissue culture cells (including ARPE-19) was in agreement with XY-plane axial length measurements for mitochondrial size [11]. Mitochondrial volumes were obtained using the “surfaces” function and manually edited on a per cell basis to acquire precise volume measurements. Fluorescent accumulation of mCherry-BAX was quantified similarly. For kinetics studies, imaged cells must have completed full BAX recruitment within the imaging interval and the entirety of the cell must have stayed within the 3-dimensional compartment defined by the Z-stack to be used for the analysis. Previously, we evaluated the BAX recruitment rates for individual BAX punctae within a cell [6]. For this study, BAX recruitment within the entire cell was combined, and therefore represents an average of the BAX recruitment kinetics for that cell. Data for BAX recruitment was then evaluated for baseline levels for each cell. All cellular datasets were then normalized to make the baseline value of BAX equal to 1.0. Plots of fluorescent intensity for each cell were then used to define the maximum rate of recruitment, the duration of recruitment, and the total amount of BAX recruited.

Still images of cytochrome c-GFP fluorescence selected from live-cell imaging sessions for presentation were processed further in Photoshop CC 2019 (Adobe, San Jose, CA) for presentation. All images along the same temporal series were modified equally to highlight mitochondrial or cytosolic localization using the exposure function to adjust exposure and offset parameters.

Statistical analysis

For comparison of two samples, we used an un-paired Student's *t*-test assuming unequal variance. For comparison of multiple samples, we used a 1-way ANOVA. For comparison of data along two linear slopes, we used a paired Student's *t*-test because each data point per sample represented a value at a fixed time point. For comparison of the frequency of imaged cells that exhibited BAX recruitment as a function of time we used a χ^2 test. Statistical significance was set to $\alpha = 0.05$.

Results

Elamipretide accelerates an increase in mitochondrial volume in cultured ARPE-19 cells

To validate that elamipretide was influencing mitochondrial dynamics in ARPE-19 cells, they were first nucleofected with a plasmid expressing mitoBFP and cultured for 24 hrs. Cells were then incubated with 1 μ M elamipretide or vehicle for 20 hrs (Fig. 1). At time points during the experiment, cells were imaged by confocal microscopy. Z-stack files were then analyzed with Imaris and the average volume of individual mitochondria was measured. Within 1 hr, cells in both groups exhibited similarly low mitochondrial volumes. By 4 and 7 hrs, however, cells exposed to elamipretide exhibited a significant increase in mitochondrial volume ($P = 0.005$ and $P = 0.002$, respectively) relative to untreated cells. By 20 hrs, both groups of cells exhibited statistically similar volume increases ($P = 0.207$) that were substantially greater than those measured 1 hr after plating.

Elamipretide does not delay the onset of BAX recruitment or permeabilization of the mitochondrial outer membrane during apoptosis

ARPE-19 cells were triple nucleofected with plasmids for CMV-mCherry-BAX, CMV-cytochrome c-GFP, and CMV-mitoBFP. After 24 hrs, fresh media was added containing vehicle or different concentrations of elamipretide ranging from 0.01 μ M to 10 μ M. Apoptosis was induced by the addition of STS to a final concentration of 1 μ M (considered time 0 min). All of the cells treated with elamipretide and STS showed the capacity to recruit and activate BAX leading to MOMP (Fig. 2). BAX recruitment was evident by the organization of labeled BAX protein moving from a diffuse cytosolic localization to form bright punctae that co-localized with mitoBFP labeled mitochondria. Additionally, 100% of the cells showing BAX translocation exhibited the release of cytochrome c-GFP (Fig. 2) that occurred rapidly at the time of initial BAX recruitment. The rapid release of these intramitochondrial proteins was also observed in vehicle treated ARPE-19 cells (data not shown) and is a typical feature of other cell types treated with STS [6].

These data showing the uninhibited capacity for BAX to be recruited to the MOM are inconsistent with a previous report showing reduced BAX accumulation in the mitochondrial fraction of cells from the basal temporal lobes of mice treated with elamipretide and exposed to a subarachnoid hemorrhage [24]. It is possible that these conflicting observations are a result of a delay in the onset of BAX recruitment induced by the drug. To assess this in our dataset, we mapped the timing of when BAX recruitment was initiated in all the cells that were imaged (Fig. 3). Previously, we reported that recruitment of BAX was not simultaneous in tissue culture cells treated with STS and that there were differences among cell types both as to when cells begin to exhibit BAX recruitment and the period of time over which a majority of cells underwent recruitment. For example, HeLa cells exhibit recruitment starting at around 2 hrs after STS addition with the majority of cells converting over a further period of 1.7 hrs. HCT116 cells, however, initiate BAX recruitment after 7 hrs of STS treatment and cells continue to recruit over a period of 5 hrs [6]. Preliminary experiments using untreated ARPE-19 cells revealed that STS treatment induced recruitment of a majority of cells during a window between 14–21 hrs so consequently most of the imaging experiments examining the effect of elamipretide were biased during this window. To evaluate if elamipretide treated cells were more or less likely to recruit BAX during this period, we used a χ^2 statistic to test if there was a difference in the proportion of cells that were imaged that converted during this window relative to imaged cells that failed to convert during this window. Data from imaging experiments that were conducted prior to 7 hrs after STS addition were excluded from this analysis. There was no significant difference between any of the treatment groups compared to vehicle treated cells, or between the combined data of the treatment groups compared to vehicle (Table 1). We interpret the results of this analysis as indicating that elamipretide had no effect in delaying the onset of BAX recruitment in response to STS in ARPE-19 cells in our imaging dataset. Further temporal analysis, which is not biased by pre-selected imaging windows of time, could address this issue in the future.

Table 1
Elamipretide does not delay the onset of BAX recruitment.

Treatment	Converting Cells in Imaging Window*	Non-converting Cells in Imaging Window**	Chi ² Statistic	P value
Vehicle	21	11		
0.01 μ M Elamipretide	7	6	0.546	0.460
0.1 μ M Elamipretide	6	9	2.743	0.098
1.0 μ M Elamipretide	15	4	1.019	0.313
10 μ M Elamipretide	5	0	0.733†	0.392
Elamipretide Combined	33	19	0.040	0.841
Table showing the total numbers of cells imaged for vehicle and treatment groups. P values shown are each individual treatment group and all elamipretide treated cells combined, compared to the vehicle treated group.				
*The imaging window used for analysis was between 14–21 hrs after the addition of STS. Converting cells denotes the number of cells being imaged that showed recruitment of BAX during this window.				
**Non-converting cells refers to the cells that were imaged but did not recruit BAX during the period. A majority of these cells went on to exhibit BAX recruitment after the imaging window, but this was not quantified relative to imaged cells that still exhibited cytosolic BAX at 24 hrs after STS addition.				
†This Chi ² test was conducted by assuming that the number of non-converted cells equaled 1. This value was not included in the summary of all the elamipretide data combined.				

Elamipretide retards the rate of BAX recruitment during apoptosis

Live cell imaging data was also analyzed to determine the maximum rate of BAX recruitment, the duration time for recruitment, and the total amount of BAX finally recruited (Fig. 4A). The maximum rate is defined as the linear slope of the stoichiometric recruitment curve [6]. Scatter plots of the mean recruitment rates for all BAX punctae formed in cells in all treatment groups are shown in Fig. 4B. No single elamipretide treatment group exhibited a significant reduction in rates compared to vehicle treated cells, although there is an apparent overall trend to lower maximal rates in the presence of elamipretide. The data was reanalyzed to combine all treatment groups during a normalized 20 minute period of recruitment and compared to the same period for vehicle treated cells. In a paired analysis elamipretide was shown to yield a significantly slower rate of BAX recruitment (Fig. 4C, $P = 6.76 \times 10^{-5}$). Assessment of the duration of the recruitment period showed a trend for longer durations in elamipretide treated cells, with 0.01 μ M elamipretide yielding significantly longer times relative to vehicle treated cells (Fig. 4D, $P =$

0.004) by an average of 9.6 minutes. When the elamipretide data was combined, the overall average duration time was 4 minutes longer for elamipretide cells compared to vehicle (Fig. 4E, $P = 0.036$). The moderate decrease in the rate of BAX recruitment, combined with the moderate increase in the duration time of recruitment resulted in no significant difference in the final amount of BAX that was recruited to cells under these conditions (Fig. 4F).

Elamipretide does not impact mitochondrial fragmentation after completion of BAX recruitment

To assess if Elamipretide also interfered with mitochondrial fragmentation, we measured the average volume of mitochondria in cells in the periods before, during, and after the recruitment of mCherry-BAX in ARPE-19 cells treated with STS (refer to Fig. 4A). There was no difference between treatment groups and vehicle treated cells in mitochondrial volume either before or during the period of BAX recruitment (ANOVA, $P = 0.942$ and $P = 0.384$, respectively). All groups showed a similar and significant decrease in average mitochondrial volume during the period immediately following BAX recruitment ($P < 0.006$).

Discussion

The effects of elamipretide on mitochondrial recovery after plating

In our experimental paradigm, ARPE-19 cells exhibited small mitochondria 24 hrs after nucleofection and plating. Over the course of an additional 20 hrs, these cells, regardless of treatment, acquired significantly larger mitochondria (as a function of average mitochondrial volume). This observation suggests that mitochondrial fusion events are predominant while adapting to the change in culture environment. Elamipretide exposure appeared to accelerate this process, with cells exhibiting greater increases in organellar volume as early as 4 hrs after addition of the peptide. These results are consistent with reports in animal studies showing that elamipretide enhances mitochondrial dynamics [29], stabilizes cristae structure, and improves oxidative phosphorylation and respiratory capacity, thereby reducing the production of reactive oxygen species [22]. Thus, we would predict that elamipretide would allow cells to recover from nucleofection and replating more rapidly than untreated cells. The observation that untreated cells eventually reached the same mitochondrial equilibrium is also not unexpected. Many of the protective and/or restorative effects of elamipretide have been reported in tissues affected by age-related changes and not in healthy cells that have reached an optimal state of mitochondrial dynamics and function [22].

Elamipretide does not phenocopy the actions of DRP1 on the recruitment and activation of BAX

Elamipretide is known to stabilize cardiolipin content and peroxidation levels in the MIM [22]. Additionally, DRP1 functions to regulate the disorganization of cristae and the transfer of cardiolipins to the MOM during apoptosis to create a lipid signaling platform that is conducive to the recruitment and organization of BAX aggregates [14]. Consequently, we hypothesized that elamipretide would phenocopy the effects of DRP1 loss of function in the process of BAX recruitment, MOMP, and mitochondrial fragmentation. Previously, we documented the effect on these events in DRP1-deficient conditions [11]. Loss of DRP1

has no effect on both the time of initiation and the maximal rate of BAX recruitment to the MOM but does reduce the overall level of BAX that is recruited. DRP1-deficiency also retards the release of cytochrome c (but not SMAC/Diablo) and reduces mitochondrial fragmentation during apoptosis. This latter effect more likely reflects the role of DRP1 in the fission complex than on its role in regulating cristae destabilization and cardiolipin transfer (although the enrichment of cardiolipins in the MOM are thought to enable increased membrane curvature in the scission sites) [14]. Elamipretide failed to induce any of these effects in our experiments. BAX recruitment, which did initiate normally, was marginally slower. This did not impact a normal MOMP resulting in the release of cytochrome c, the total amount of BAX recruited to mitochondria, or the ability of the mitochondria to undergo fragmentation. These results imply that elamipretide works by an independent mechanism from DRP1, which is consistent with studies showing that elamipretide and the DRP1 inhibitor Mdivi1 work synergistically to reduce mitochondrial dysfunction in neurons expressing mutant amyloid β precursor protein [25].

The mechanism of elamipretide on slowing the rate of BAX recruitment is not known. One possibility is that elamipretide moderately reduces cardiolipin transfer from the MIM to the MOM, thereby making the lipid signaling platform in the MOM less conducive to BAX integration. This hypothesis remains to be tested but seems unlikely based on our evidence that the release of cytochrome c, which is affected by cardiolipin transfer, is unabated by the drug. An alternate hypothesis is that BAX recruitment is impaired by Parkin-dependent ubiquitination [30]. Elamipretide is known to increase the levels of mitophagy [31], which implies an increase in Parkin activity. Currently there is little evidence that elamipretide affects Parkin activity, although a study of the effect of this peptide on swine kidney senescence shows that prolonged systemic delivery of the drug is able to increase Parkin levels and localization to mitochondria in a model of atherosclerotic renal artery stenosis [32]. This is an interesting correlation that warrants further study. Overall, our data suggest that the observed protective effect of elamipretide is likely upstream of BAX activation, and not a function of inhibiting this step in the apoptotic pathway. To our knowledge, the effect of elamipretide on the transcriptome of cells has not been studied, but it is possible that this peptide leads to modulation of the upstream signaling of different BH3-only proteins. Most cells express, and/or activate, a spectrum of BH3-only proteins, each of which has different affinities for anti- and pro-apoptotic members of the BCL2 gene family [33]. Changing this spectrum could conceivably affect the kinetics of BAX activation. This possibility remains to be tested in cells treated with elamipretide.

Limitation

Studies measuring the kinetics of BAX recruitment and activation were done on tissue culture cells treated with the non-specific kinase inhibitor staurosporine, which may not necessarily accurately reflect the apoptotic signaling of cells in a complex tissue environment.

Abbreviations

BH and BH3, BCL2 homology domain, and BCL2 homology domain 3

MIM, mitochondrial inner membrane

MOM, mitochondrial outer membrane

MOMP, mitochondrial outer membrane permeabilization

STS, staurosporine

Declarations

Author's contributions

RN, DK, and MK developed the experimental design, which was refined further by JG. JG and RF conducted the experiments under the supervision of RN. JG and RN analyzed the data and wrote the manuscript, which was revised with input by all the co-authors.

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Availability of data and materials

Raw data, including imaging files, and reagents described in this study will be made available upon request to the corresponding author. Elamipretide was provided by Stealth BioTherapeutics, Inc., and requests for that reagent should be made directly to the company.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

Funding and reagent (elamipretide) for this study was provided by Stealth BioTherapeutics, Inc. Drs. Dennis Keefe and Martin Redmond are employees of Stealth BioTherapeutics, Inc.

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Figures

Figure 1

Elamipretide stimulates a more rapid increase in mitochondrial volume. Mitochondria were identified in ARPE-19 cells nucleofected with a plasmid carrying a mitoBFP fusion protein. Nucleofected cells were plated in chamber slides, allowed to incubate for 24 hrs and then imaged for another 20 hours. (A) Confocal image of a cell imaged at 1 hr and then again at 7hrs (B) after exposure to 1 μM elamipretide. Only the BFP channel is shown. Over time, mitochondria appear more filamentous and elongated. Size bar=10 μm . (C) Quantification of average mitochondrial volumes of cells taken from both time-lapse and static images. The scatterplot shows data collected from individual cells at each time point (mean $\pm$ SD also indicated). Mitochondrial volume was measured in 3D reconstructions of confocal images using Imaris 9.2 imaging software. Elamipretide treated cells exhibit significantly greater mitochondrial volumes at 4 and 7 hrs after exposure to the peptide (* $P=0.005$ and ** $P=0.002$, respectively). By 20 hrs, both treatment groups exhibit similarly larger mitochondria ($P=0.207$).

Figure 2

Time lapse imaging stills of an ARPE-19 cells undergoing apoptosis. The stills shown are of a cell treated with 0.01 μM elamipretide and 1 μM staurosporine. This cell was nucleofected with plasmids expressing mCherry-BAX, cytochrome c-GFP, and mitoBFP fusion proteins. The images just prior to the initiation of BAX recruitment are shown (A, E, I, M), along with +2 min, +4 min, and +26 min after this time stamp. Some BAX puncta are evident just prior to the declaration of BAX recruitment (E) and it is not clear if these are activated BAX aggregates, or excess fusion protein that is sequestered in lysosomes [6]. Once BAX recruitment is initiated, cytochrome c moves from a mitochondrial localization (I) to a diffuse cytosolic localization (J-L). Mitochondria remain intact through the initial stages of BAX recruitment (M-O) but become rapidly fragmented and difficult to detect shortly after BAX becomes fully recruited (D, H, L, P).

P). These results are typical for both vehicle and elamipretide treated cells. Images for cytochrome c-GFP have been modified to enhance exposure levels. Size bar=7 μm .

Figure 3

Temporal assessment of cells undergoing mCherry-BAX recruitment during live-cell imaging experiments. Data collected from 13 imaging experiments are graphed. Each point represents a single cell. The horizontal lines indicate the duration of the imaging session and the points on each line indicate cells that underwent BAX recruitment during that session. Points represented in the column labeled “Other” represent cells that were set up for imaging but did not convert cytosolic BAX to punctate BAX.

Figure 4

Kinetic analysis of mCherry-BAX recruitment in the presence of elamipretide. (A) A typical sigmoid BAX recruitment curve showing various metrics that were obtained, including the maximum rate and the times for initiation and end of BAX accumulation, which are used to determine the duration of the recruitment period. The plateau of the recruitment curve is used to determine the total accumulation of BAX being recruited. This curve represents individual BAX punctae, which are then averaged for the entire cell being

examined. (B) Scatter plot of calculated maximal recruitment rates for cells analyzed in each treatment group. (C) Combined BAX recruitment data for vehicle (black circles) plotted against all elamipretide groups combined (grey circles). The data used in this analysis represents the first 20 minutes of recruitment for all cells that were successfully imaged and analyzed. The combined data for elamipretide shows a significantly slower rate of recruitment ($P=6.76 \times 10^{-5}$, paired t-test). (C) Duration periods for all treatment groups. Treatment with 0.01 μM elamipretide yielded significantly longer duration periods compared to vehicle treated cells ($*P=0.004$). (E) Bar graph showing duration times of vehicle treated cells against the combined elamipretide treated groups. Durations are significantly longer in the presence of elamipretide ($*P=0.036$). (F) Bar graph showing the total amount of BAX recruited in vehicle treated cells relative to the combined elamipretide treated groups. No significant difference was detected.

Figure 5

Analysis of apoptotic mitochondrial fragmentation in the presence of elamipretide. Scatter plot of average mitochondrial volume of ARPE-19 cells treated with vehicle or different concentrations of elamipretide. Mitochondrial volumes were calculated from time lapse confocal images of cells expressing mitoBFP to label mitochondria. Average volumes were calculated at least 20 minutes prior to the onset of mCherry-BAX recruitment (Phase 1), during the period of mCherry-BAX recruitment (Phase 2), and during a 20 minute interval after mCherry-BAX recruitment (Phase 3) (see Figure 4A). ANOVA analysis showed no difference in volumes among the treatment groups in each phase ($P \geq 0.38$). All treatment groups showed a significant decrease in mitochondrial volume in Phase 3 relative to the other Phases ($*P \leq 0.0018$).