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Synergistic Protective Effects of Mitochondrial Division Inhibitor 1 and Mitochondria-Targeted Small Peptide SS31 in Alzheimer's Disease

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Abstract

The purpose of our study was to determine the synergistic protective effects of mitochondria-targeted antioxidant SS31 and mitochondria division inhibitor 1 (Mdivi1) in Alzheimer's disease (AD).

Using biochemical methods, we assessed mitochondrial function by measuring the levels of hydrogen peroxide, lipid peroxidation, cytochrome c oxidase activity, mitochondrial ATP and GTPase Drp1 enzymatic activity in mutant A β PP cells. Using biochemical methods, we also measured cell survival and apoptotic cell death. Amyloid beta (A β) levels were measured using sandwich ELISA, and using real-time quantitative RT-PCR, we assessed mtDNA (mtDNA) copy number in relation to nuclear DNA (nDNA) in all groups of cells. We found significantly reduced levels of A β 40 and A β 42 in mutant A β PP cells treated with SS31, Mdivi1 and SS31+Mdivi1, and

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the reduction of A β 42 levels were much higher in SS31+Mdivi1 treated cells than individual treatments of SS31 and Mdivi1. The levels of mtDNA copy number and cell survival were significantly increased in SS31, Mdivi1 and SS31+Mdivi1 treated mutant A β PP cells, however, the increased levels of mtDNA copy number and cell survival were much higher in SS31+Mdivi1 treated cells than individual treatments of SS31 and Mdivi1. Mitochondrial dysfunction is significantly reduced in SS31, Mdivi1 and SS31+Mdivi1 treated mutant A β PP cells, however the reduction is much higher in cells treated with both SS31+Mdivi1. Similarly, GTPase Drp1 activity is reduced in all treatments, but reduced much higher in SS31+Mdivi1 treated cells. These observations strongly suggest that combined treatment of SS31+Mdivi1 is effective than individual treatments of SS31 and Mdivi1. Therefore, we propose that combined treatment of SS31+Mdivi1 is a better therapeutic strategy for AD. Ours is the first study to investigate combined treatment of mitochondria-targeted antioxidant SS31 and mitochondrial division inhibitor 1 in AD neurons.

Keywords

Mitochondrial division inhibitor 1; Mitochondria-targeted antioxidant SS31; Amyloid- β ; Mitochondrial dysfunction; Mitochondrial dynamics; Mitochondrial fission; Synergistic protective effects; Mitochondrial biogenesis

Introduction

Alzheimer's disease is a progressive, neurodegenerative disorder manifested by dementia in aged individuals [1]. Currently, over 46.8 million people worldwide live with AD-related dementia, and this number is expected to increase to 131.5 million by 2050 [2]. AD-related dementia has huge economic consequences, with the total worldwide medical cost of dementia in 2015 estimated at \$818 billion. By 2018, AD, including AD-related dementia, is expected to become a trillion-dollar disease [2]. In addition to dementia, AD is associated with the multiple cellular changes, including loss of synapses, synaptic dysfunction, mitochondrial structural and functional abnormalities, inflammatory responses, neuronal loss, extracellular neuritic plaques, and intracellular NFTs [3–8]. Despite the progress that has been made in better understanding of AD pathogenesis, we still do not have early detectable markers, drugs/agents that can prevent AD or slow its progression.

Several decades of intense research have revealed that mitochondrial dysfunction and synaptic damage are early events in the AD disease process. Mitochondria play several key roles, including synaptic maintenance, ATP generation, intracellular Ca²⁺ regulation, free radical production and scavenging and activation caspase family of proteases [9]. Regarding ATP generation, it has long been recognized that neuronal activity can stimulate oxidative phosphorylation. Several studies found increased free radical production, lipid peroxidation, oxidative DNA and protein damage, and decreased mitochondrial ATP production in postmortem AD brains, compared to postmortem healthy brains [10–12]. Further, using AD transgenic mice lines, several studies have found increased production of free radicals and lipid peroxidation, and reduced levels of cytochrome c oxidase activity and ATP in AD-affected brain regions (cortex and hippocampus) of patients with AD [13–21], in the primary neurons of AD transgenic mice, and in neurons expressing mutant A β PP and A β [22–26].

These findings strongly implicate mitochondrial dysfunction and oxidative stress in AD pathogenesis.

Several recent studies reported that mutant A β PP and A β are associated with Mitochondria [5]. A β monomers and oligomers were found in mitochondrial membranes in neurons of postmortem specimens from AD patients and AD mice brains, and in neurons expressing mutant A β PP [15,27–30]. Findings from Hanson Peterson et al. (2008) [30] together with those from Manczak et al. (2006) [15], Crouch et al. (2005) [28], Caspersen et al. 2005 [27], and Devi et al. (2006) [10] suggest that A β species transported to mitochondrial membranes cause impaired mitochondrial dynamics, excessive fragmentation of mitochondria, mitochondrial dysfunction and oxidative damage, and ultimately damage to AD neurons both structurally and functionally. These studies strongly suggest that mitochondria-targeted molecules and/or mitochondrial division inhibitors are possible candidates to reduce mitochondria-generated free radicals and also to reduce excessive fragmentation of mitochondria in AD neurons.

Recently, several mitochondria-targeted molecules have been developed and currently being tested in several laboratories [31–32]. SS31 is one of the cell permeable, tetra-peptides synthesized with other SS peptides (SS02, SS19, SS20) [33–34]. SS31 has been found to target mitochondria and to protect neurons from Ca²⁺-induced mitochondrial depolarization swelling, to release cytochrome c in isolated organelles, and to protect cells against various mitochondrial insults. SS31 was found to inhibit lipid peroxidation and hydrogen peroxide scavenging [34]. SS31 was also evaluated in rat models of islet cell transplantation [35], myocardial infarction [36], and ALS [37]. Strikingly, SS31 was beneficial in reducing ROS all of these studies. Based on the neuroprotection capacity of SS31 in the rat model, we evaluated SS31 in cell models of AD, including A β -treated mouse neuroblastoma (N2a) cells and primary neurons from A β PP mice [22]. We found SS31 reduces mitochondrial dysfunction and oxidative stress both of which are known characteristics of A β -induced toxicities in AD [22]. We also found SS31 treatment of primary neurons reversed the impaired trafficking of mitochondrial and reduced excessive mitochondrial fission and increased mitochondrial fusion. Further, mitochondrial function and synaptic activities were increased in A β PP primary neurons treated with SS31 [24]. These observations suggest that SS31 is beneficial to AD neurons.

To identify mitochondrial fission inhibitors, several research groups have independently screened chemical libraries and have found several mitochondrial fission inhibitors: Mdivi1 [38], Dynasore [39], and P110 [40]. Among these, Mdivi 1 has been extensively studied with experimental rodent models of epilepsy [41–42], ischemia and reperfusion injury [43–45], oxygen glucose deprivation [46]. In these diseased states and conditions, Mdivi1 was found to reduce excessive mitochondrial fission, to maintain mitochondrial fission-fusion balance, and to maintain the normal functioning of cells. Mdivi1 was also found to inhibit GTPase Drp1 activity by blocking the self-assembly of Drp1, resulting in the irreversible formation of elongated and tubular mitochondrial in WT cells [38]. Recently, using molecular, cell biology and biochemical methods, we studied the protective effects of Mdivi1 against A β - and mitochondrial fission protein, Drp1-induced excessive mitochondrial fragmentation in AD [47]. We also studied preventive (Mdivi1+A β 42) and curative (A β 42+Mdivi1) effects

against A β 42 in N2a cells. A β 42 was found to impair mitochondrial dynamics, lowered mitochondrial biogenesis and mitochondrial function and lowered synaptic activity. On the contrary, Mdivi1 enhanced mitochondrial fusion activity, lowered fission machinery, and increased biogenesis and synaptic proteins. Mitochondrial function and cell viability were elevated in Mdivi1-treated cells [47]. However, the synergistic protective effects of SS31 and Mdivi1 in AD neurons is not studied yet.

The purpose of our current study is to investigate the synergistic beneficial effects of SS31+Mdivi1 using 1) mouse neuroblastoma (N2a) cells and 2) N2a cells transfected with mutant A β PP cDNA (Swedish and Indiana mutations), referred as mutant A β PP cells. In our initial investigation, we studied the following: mtDNA copy number relative to nuclear DNA, cell survival and apoptotic cell death, mitochondrial function and GTPase Drp1 enzymatic activity in N2a cells and mutant A β PP cells treated with SS31, Mdivi1 and SS31+Mdivi1 relative to untreated mutant A β PP cells. In mutant A β PP cells treated with SS31, Mdivi1 and SS31+Mdivi1, we also assessed A β 40 and A β 42 levels.

Materials and Methods

Chemicals and Reagents

Mdivi1 was purchased from Sigma-Aldrich Chemical Company (City, CA, USA); SS31 was synthesized and purchased from Biogenix, Inc., and N2a cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). Dulbecco's Modified Eagle Medium (DMEM) and Minimum Essential Medium (MEM), penicillin/streptomycin, Trypsin-EDTA, and fetal bovine serum were purchased from GIBCO (Gaithersburg, MD, USA).

Mutant A β PP cDNA construct

We purchased mutant A β PP cDNA clone (pCAX-A β PP Swe/Ind) from Addgene - <https://www.addgene.org> and further sub-cloned into a mammalian expression vector pRP-Puro-CAG. pRP vector is pUC backbone having CMV promoter and SV40 polyadenylation site with puromycin selection for stable transfection. The sequence output was confirmed with NCBI sequence hA β PP [NM_201414.2]*(K595N M596L V642F). Expression of mutant A β PP cDNA was verified for A β PP mutant protein expression. We transfected mutant A β PP cDNA into mouse neuroblastoma (N2a) cells using lipofectamine 3000 for 24 hrs. After transfection, cells were treated with SS31, Mdivi1 and in combination of SS31+Mdivi1 for 6 hrs. We harvested mutant A β PP cells treated and untreated with SS31, Mdivi1 and in combination of SS31+Mdivi1 for further analysis.

Tissue culture work

The N2a cells were grown for 3 days in a medium (1:1 mixture of DMEM and OptiMEM, 10% FBS plus penicillin and streptomycin [Invitrogen, Carlsbad, CA, USA]) until the cells are confluent. As shown in Figure 1, these cells were used for 5 groups - one control group and 4 treatment groups: 1) untreated N2a cells (the control group), 2) N2a cells transfected with mutant A β PP cDNA with Swe and Indiana mutations, 3) N2a cells transfected with mutant A β PP+treated with SS31 peptide for 6 hrs, 0.25nM final concentration, 4) N2a cells

transfected with mutant A β PP+ treated with Mdivi1 for 6 hrs, 20 μ M final concentration, 5) N2a cells transfected with mutant A β PP cDNA+SS31+Mdivi1. As shown in Figure 1, we performed 4 independent cell cultures and transfections with mutant A β PP cDNA treatments for all experiments (n=4).

To determine the protective effects of SS31, Mdivi1 individually and SS31+Mdivi1 together in N2a cells, we also treated N2a cells with SS31, N2a cells with Mdivi1 and SS31+Mdivi1 together. We assessed 1) mtDNA copy number to nDNA, 2) levels of cell survival and apoptotic cell death, 3) mitochondrial function and 4) GTPase Drp1 activity in treated with SS31, Mdivi1 and SS31+Mdivi1 relative to untreated N2a cells. The concentrations of Mdivi1 and SS31 were optimized in our laboratory based on serial dilutions in N2a cells (unpublished data).

The N2a cells from all groups were harvested, and their cell pellets were collected and used for mitochondrial functional assays by measuring the levels of hydrogen peroxide, lipid peroxidation, and cytochrome c oxidase activity. Using biochemical methods, we also assessed cell viability and cell survival and apoptosis cell death. We also assessed GTPase Drp1 enzymatic activity using Drp1-immunoprecipitation elutes. A β levels were measured using sandwich ELISA assay. Mitochondrial ATP was measured from isolated mitochondria in cells from all groups. As shown in Figure 1, we performed 4 independent cell cultures and treatments for all parameters. Detailed methods are given below:

Immunoblotting analysis—Immunoblotting analysis was performed using protein lysates prepared from N2a transfected and transfected mutant A β PP cDNA using 6E10 antibody that recognizes mutant full-length A β PP and A β as described in Manczak et al, 2010 (22). Twenty μ g protein lysates were resolved on a 4–12% Nu-PAGE gel (Invitrogen). The resolved proteins were transferred to nylon membranes (Novax Inc., San Diego, CA, USA) and were then incubated for 1 hour at room temperature with a blocking buffer (5% dry milk dissolved in a TBST buffer). The nylon membranes were incubated overnight with the primary antibody (6E10-1:500 monoclonal, BioLegend, San Diego, CA). The membranes were washed with a TBST buffer 3 times at 10-minute intervals and were then incubated for 2 hours with appropriate secondary antibody Sheep anti-mouse HRP 1:10,000, followed by 3 additional washes at 10-minute intervals. Proteins were detected with chemiluminescence reagents (Pierce Biotechnology, Rockford, IL, USA), and the bands from immunoblots were visualized.

Immunofluorescence analysis and quantification—To determine mitochondrial network, immunofluorescence analysis was performed using mutant A β PP cells and untransfected N2a and TOM20 antibody as described in Manczak et al, 2010 (22). The fixed cells were washed with warm PBS, fixed in freshly prepared 4% paraformaldehyde in PBS for 10 minutes, and then washed with PBS and permeabilized with 0.1% Triton-X100 in PBS. They were blocked with a 1% blocking solution (Invitrogen) for 1 hour at room temperature. All sections were incubated overnight with TOM20 antibody (1:250 dilution). After incubation, the cells were washed 3 times with PBS, for 10 minutes each. The cells were incubated with a secondary antibody conjugated with Fluor 488 (Invitrogen) for 1 hour at room temperature. The cells were washed 3 times with PBS and mounted on slides.

Photographs were taken with a multiphoton laser scanning microscope system (ZeissMeta LSM510). To quantify the immunoreactivity of TOM20 antibody for each treatment, 10–15 photographs were taken at $\times 40$ magnifications, and statistical significance was assessed, using one-way ANOVA for TOM20 protein.

Apoptosis assay—Cell based apoptosis assay was performed using Cellometer Vision CBA Image Cytometry System (Nexcelom Bioscience LLC, Lawrence, MA) with two fluorophore Annexin V-FITC and Propidium Iodide (PI) staining solution, according to manufacturer's instructions. Briefly, cells were harvested using trypsin, then spin down at 300g for three minutes and pellets were washed with 1XPBS, cells were counted using hemacytometer. Collected 100,000 to 150,000 cells and cells/pellet was resuspended in 40 μ l of Annexin V binding buffer. 5 μ l each of Annexin V –FITC reagent (green) and PI (red) were added to binding buffer containing cells; gently mix solution by pipetting up and down ten times, then incubate for 15 min at RT in the dark; after incubation, add 250 μ l of 1XPBS and spin down at 300g for three minutes, then re-suspended the cell pellets in 50 μ l of Annexin V binding buffer, then assess the cells apoptosis. Gate purple represents live cells, gate green represents the positive apoptotic cells, gate blue represents for the detection of positive necrotic cells and gate red represents debris.

Mitochondrial DNA copy number relative to nuclear DNA copy number—To determine the mitochondrial DNA copy number, we measured mtDNA to nDNA ratios using NovaQUANT Kit from Novagen (EMD Millipore Corporation, San Diego, CA). In this assay, copy numbers of 2 mitochondrial genes – NEB and BECN1 and 2 nuclear genes 12S and TrLEV were assessed. Real-Time quantitative PCR was conducted according to manufacturer's instructions. 2ng DNA was used for each reaction, real-time PCR was performed using the Applied Biosystems 7900HT fast real time PCR system. The ratios of mtDNA to nDNA were calculated using the calculation $N = (2^{-Ct1} + 2^{-Ct2})/2$, where $Ct1 = Ct(BECN1) - Ct(trLEV)$, and $Ct2 = Ct(NEB) - Ct(12S)$. Based on these observations, we calculated mtDNA copy number.

Measurement of soluble A β levels—Soluble A β levels were conducted using sandwich ELISA as described in Manczak et al. 2016 [48]. Briefly, protein lysates were from cell pellets in a Tris-buffered saline (pH 8.0) containing protease inhibitors (20 mg/ml pepstatin A, aprotinin, phosphoramidon and leupeptin; 0.5 mMphenylmethanesulfonyl fluoride and 1 mM ethyleneglycol-bis(aminomethyl ether)-NN tetraacetic acid). Samples were sonicated briefly and centrifuged at 10 000g for 20 min at 4°C. The soluble fraction was used to determine the soluble A β by ELISA. For each sample, A β 1-40 and A β 1-42 were measured with commercial colorimetric ELISA kits (Biosource International, Camarillo, CA, USA) specific for human. A 96-well plate was used, following the manufacturer's instructions. Each sample was run in duplicate. Protein concentrations of the homogenates were determined following the BSA method, and A β was expressed as pg A β /mg protein.

Mitochondrial functional and GTPase Drp1 enzymatic activity assays—Mitochondrial function was assessed by measuring hydrogen peroxide, lipid peroxidation,

cytochrome c oxidase activity and ATP in N2a cells, N2a cells+SS31, N2a cells+Mdivi1 and N2a cells+SS31+Mdivi1.

Mitochondrial function was also assessed in mutant A β PP cells treated with SS31, Mdivi1 and SS31+Mdivi1. Mitochondrial functional data were compared in 2 ways – in comparison 1 - untreated/untransfected N2a cells with mutant A β PP cells, mutant A β PP cells treated with SS31, Mdivi1, SS31+Mdivi1, and in comparison 2 - mutant A β PP cells with mutant A β PP cells+SS31, Mdivi1, and SS31+Mdivi1. As shown in Figure 1, we performed 4 independent culture experiments for all mitochondrial parameters.

Hydrogen peroxide production—Using an Amplex® Red Hydrogen Peroxide Assay Kit (Molecular Probes, Eugene, OR, USA), the production of hydrogen peroxide was measured using cell pellets from groups of cells as described in Reddy et al. 2016 [49] (Figure 1). Briefly, hydrogen peroxide production was measured in the mitochondria cortical tissues from all 4 lines of mice. A BCA Protein Assay Kit (Pierce Biotechnology) was used to estimate protein concentration. The reaction mixture contained mitochondrial proteins ($\mu\text{g}/\mu\text{l}$), Amplex Red reagents (50 μM), horseradish peroxidase (0.1 U/ml), and a reaction buffer (1X). The mixture was incubated at room temperature for 30 minutes, followed by spectrophotometer readings of fluorescence (570 nm). Finally, hydrogen peroxide production was determined, using a standard curve equation expressed in nmol/ μg mitochondrial protein.

Lipid peroxidation assay—Lipid peroxidates are unstable indicators of oxidative stress in the brain. The final product of lipid peroxidation is 4-hydroxy-2-nonenol (HNE), which was measured in all groups of cells (Figure 1) We used HNE-His ELISA Kit (Cell BioLabs, Inc., San Diego, CA, USA) as described in Manczak and Reddy 2012 [50]. Briefly, freshly prepared protein as added to a 96-well protein binding plate and incubated overnight at 4°C. It was then washed 3 times with a buffer. After the last wash, the anti-HNE-His antibody was added to the protein in the wells, which was then incubated for 2 hours at room temperature and was washed again 3 times. Next, the samples were incubated with a secondary antibody conjugated with peroxidase for 2 hours at room temperature, followed by incubation with an enzyme substrate. Optical density was measured (at 450nm) to quantify the level of HNE.

Cytochrome c oxidase activity—Cytochrome c oxidase activity was measured in all groups of cells. Enzyme activity was assayed spectrophotometrically using a Sigma Kit (Sigma–Aldrich) following manufacturer’s instructions. Briefly, 2 μg protein lysate was added to 1.1 ml of a reaction solution containing 50 μl 0.22 mM ferricytochrome *c* fully reduced by sodium hydrosulphide, Tris–HCl (pH 7.0), and 120 mM potassium chloride. The decrease in absorbance at 550 mM was recorded for 1-min reactions at 10-sec intervals. Cytochrome c oxidase activity was measured according to the following formula: mU/mg total mitochondrial protein = (A/min sample – (A/min blank) $\times$ 1.1 mg protein $\times$ 21.84). The protein concentrations were determined following the BCA method.

ATP levels—ATP levels were measured in isolated mitochondria from all groups of cells using ATP determination kit (Molecular Probes). The bioluminescence assay is based on the

reaction of ATP with recombinant firefly luciferase and its substrate luciferin. Luciferase catalyzes the formation of light from ATP and luciferin. It is the emitted light that is linearly related to the ATP concentration, which is measured with a luminometer. ATP levels were measured from mitochondrial pellets using a standard curve method.

GTPase Drp1 enzymatic activity—Using a calorimetric kit (Novus Biologicals, Littleton, CO, USA), GTPase Drp1 enzymatic activity was measured in all groups of cells using Drp1-immunoprecipitation elutes following GTPase assay methods described in Manczak and Reddy 2015 [51], based on GTP hydrolyzing to GDP and to inorganic Pi. GTPase activity was measured, based on the amount of Pi that the GTP produces. By adding the ColorLock Gold (orange) substrate to the Pi generated from GTP, we assessed GTP activity, based on the inorganic complex solution (green). Colorimetric measurements (green) were read in the wavelength range of 650 nm.

Statistical considerations

Statistical analyses were conducted for all the parameters in the N2a cells from the 5 experimental groups, using one-way ANOVA with Dunnett correction. The parameters included hydrogen peroxide cytochrome c oxidase activity, lipid peroxidation, mitochondrial ATP production, cell viability, GTPase Drp1 activity, cell survival, cell death, A β levels and mtDNA copy number.

Results

Immunoblotting analysis

To determine mutant A β PP cDNA transfection in N2a cells, we conducted immunoblotting analysis of N2a cells transfected with mutant A β PP cDNA using 6E10 antibody that recognizes mutant full-length A β PP and A β . As shown in Figure 2, we found 110 kDa mutant A β PP band in N2a cells transfected with mutant A β PP cDNA, but not in untransfected N2a cells, indicating that mutant A β PP cDNA transfection was successful.

Levels of A β 42

To determine whether SS31 and Mdivi1 lowers A β 40 and A β 42 levels separately and in combination in mutant A β PP cells, using Sandwich ELISA, we measured A β 40 and A β 42 levels in mutant A β PP cells treated with SS31, Mdivi1 and SS31+Mdivi1.

As shown in Figure 3A, significantly reduced levels of A β 40 were found in the mutant A β PP cells treated with SS31 ($P=0.001$) and with Mdivi1 ($P=0.003$) and in combination treatment of Mdivi1+SS31 ($P=0.004$) compared to mutant A β PP cells. Significantly lower levels of A β 42 were found in the mutant A β PP cells treated with SS31 ($P=0.004$) and with Mdivi1 ($P=0.03$) and in combination treatment of Mdivi1+SS31 ($P=0.002$) compared to mutant A β PP cells (Figure 3B). It is interesting to note that A β 42 levels were significantly reduced in the mutant A β PP cells treated with both SS31+Mdivi1 compared to individual treatments of SS31 and Mdivi1 – in other words combination treatment reduced A β 42 levels much higher than individual treatments of Mdivi1 and SS31 in mutant A β PP cells. Overall,

these observations revealed that a combination treatment of Mdivi1+SS31 is effective than individual treatments of Mdivi1 and SS31.

Mitochondria DNA copy number in relation to Nuclear DNA

To determine the effect of SS31, Mdivi1 and combination of SS31 and Mdivi1 treatments on mtDNA to nDNA, we measured DNA copy numbers of 2 nuclear genes and 2 mitochondrial genes using NovaQuant Kit.

Significantly increased mtDNA copy number was found in SS31-treated N2a cells ($P=0.01$), Mdivi-treated N2a cells ($P=0.03$) and SS31+Mdivi1-treated N2a cells ($P=0.002$) relative to untreated N2a cells (Figure 4A).

Significantly decreased mtDNA copy number/cell in mutant A β PP cells was found ($P=0.001$) relative to untransfected N2a cells with mutant cDNA (Figure 4B). Interestingly, mtDNA copy number was increased in mutant A β PP cells treated with SS31, Mdivi1 and SS31+Mdivi1, but not significant. These observations indicate that mutant A β PP reduces mtDNA copy number, on the other hand SS31, Mdivi1 and combination treatment of SS31+Mdivi1 increases mtDNA copy number.

We also compared the data between mutant A β PP cells with mutant A β PP cells treated SS31, Mdivi1 and combination treatment of SS31+Mdivi1. We found mtDNA copy number is significantly increased in mutant A β PP cells treated with SS31 ($P=0.01$), Mdivi1 ($P=0.01$) and SS31+Mdivi1 ($P=0.005$) (Figure 4C) relative to mutant A β PP cells. However, the increased levels of mtDNA copy number is much higher for combination treatment of Mdivi1+SS31 than individual treatments of SS31 and Mdivi1 (Figure 4C). These observations strongly indicate that combined treatment is effective than individual treatments.

Immunostaining analysis of TOM20

To determine mitochondrial network in mutant A β PP cells treated with SS31, Mdivi1 and combination treatment of SS31+Mdivi1, we performed immunostaining analysis using TOM20 antibody that determines the mitochondrial network. As shown in Figure 5, we found significantly increased mitochondrial network in mutant A β PP cells treated with SS31 ($P=0.01$), Mdivi1 ($P=0.02$) and SS31+Mdivi1 ($P=0.004$) relative to untransfected mutant A β PP cells. These observations indicate that combined treatment showed increased mitochondrial network than individual treatments of SS31 and Mdivi1.

Cell survival and apoptotic cell death

To determine the effect of SS31, Mdivi1 and Mdivi1 treatment of N2a cells, we assessed cell survival and apoptotic cell death. As shown in Figure 6A, we found significantly increased cell survival in SS31 ($P=0.02$), Mdivi1 ($P=0.02$) and SS31+Mdivi1 ($P=0.004$) relative to untreated N2a cells. Significantly reduced apoptotic cell death was observed in SS31 ($P=0.002$), Mdivi1 ($P=0.002$) and SS31+Mdivi1 treated N2a cells ($P=0.001$) relative to untreated N2a cells (Figure 6B).

To determine the effect of SS31, Mdivi1 and in combination of SS31+Mdivi1 treatment of N2a cells transfected mutant A β PP cDNA (mutant A β PP cells), we assayed cell survival and apoptotic cell death. As shown in Figure 6C, we found significantly reduced cell survival in mutant A β PP cells relative to untransfected N2a cells ($P=0.005$). On the other hand, mutant A β PP cells treated with SS31, Mdivi1 and SS31+Mdivi1 showed increased cell survival relative to untreated mutant A β PP cells, but not significant (Figure 6C). These observations suggest that mutant A β PP cells decrease cell survival rates.

When we compared the data between mutant A β PP cells treated with SS31, Mdivi1 and SS31+Mdivi1 relative to mutant A β PP cells – we found significantly increased levels of cell survival in mutant A β PP cells treated with SS31 ($P=0.004$), Mdivi1 ($P=0.002$) and SS31+Mdivi1 ($P=0.001$) (Figure 6D). These observations strongly suggest combination treatment is better than individual treatment of SS31 and Mdivi1.

As shown in Figure 6E, we found significantly increased apoptotic cell death in mutant A β PP cells relative to untransfected N2a cells ($P=0.001$). On the other hand, apoptotic cell death was significantly decreased in mutant A β PP cells treated with SS31 ($P=0.003$), Mdivi1 ($P=0.04$) and SS31+Mdivi1 ($P=0.001$) relative to untreated mutant A β PP (Figure 6E).

Significantly decreased levels of apoptotic cell death in mutant cells treated SS31 ($P=0.002$), Mdivi1 ($P=0.001$) and SS31+Mdivi1 ($P=0.0001$) relative to untreated mutant A β PP cells (Figure 6F). These observations indicate that mutant A β PP is toxic to cells and SS31, Mdivi1 and combined treatment of SS31 and Mdivi1 is protective against mutant A β PP and A β to cells.

Mitochondrial function and GTPase Drp1 activity

Parameters of mitochondrial function and GTPase Drp1 activity were studied in N2a cells treated with SS31, Mdivi1 and combined treatment of SS31+Mdivi1 relative to untreated N2a cells ($n=4$) in order to determine whether SS31, Mdivi1 separately and/or in combination maintains mitochondrial function and GTPase Drp1 activity. The parameters included hydrogen peroxide production, cytochrome c oxidase activity, lipid peroxidation (4-hydroxynonenal) and ATP production and GTPase Drp1 activity.

N2a cells treatment with SS31, Mdivi1 and SS31+Mdivi1

Hydrogen peroxide production: Significantly reduced levels of hydrogen peroxide were found in SS31 ($P=0.01$), Mdivi1 ($P=0.03$) and SS31+Mdivi1 ($P=0.004$) treated N2a cells relative to untreated N2a cells (Figure 7A).

Lipid peroxidation: As shown Figure 7B, significantly reduced levels of lipid peroxidation were found in SS31 ($P=0.03$), Mdivi1 ($P=0.04$) and SS31+Mdivi1 ($P=0.01$) treated N2a cells relative to untreated N2a cells.

Cytochrome c oxidase activity: Significantly increased levels of cytochrome c oxidase were found in SS31 ($P=0.02$), Mdivi1 ($P=0.03$) and SS31+Mdivi1 ($P=0.004$) treated N2a cells relative to untreated N2a cells (Figure 7C).

ATP production: As shown in Figure 7D, significantly increased levels of ATP were found in SS31 (P=0.02), Mdivi1 (P=0.02) and SS31+Mdivi1 (P=0.004) treated cells relative to untreated N2a cells.

GTPase Drp1 activity: As shown in Figure 8A, we found GTPase Drp1 activity was significantly decreased in SS31-treated N2a cells (P=0.02), Mdivi1 treated N2a cells (P=0.03) and SS31+Mdivi1 treated N2a cells (P=0.01).

N2a cells transfected with mutant A β PP cDNA and treatments with SS31, Mdivi1 and SS31+Mdivi1—To determine the effects of mutant A β PP and protective effects of SS31, Mdivi1 and combined treatments of SS31+Mdivi1 cells, we assessed mitochondrial function and GTPase Drp1 activity in 5 groups of cells – 1. untreated/untransfected N2a cells, 2. cells transfected with mutant A β PP, 3. Mutant A β PP cells treated with SS31, 4. Mutant A β PP cells treated with Mdivi1 and 5. Mutant A β PP cells treated with SS31+Mdivi1. We compared the data 2 ways – in comparison 1 – untransfected N2a cells with mutant A β PP cDNA with the treatments SS31, Mdivi1, and SS31+Mdivi1, and in comparison 2 – mutant A β PP cDNA transfected N2a cells (mutant A β PP cells) with the treatments of SS31, Mdivi1 and SS31+Mdivi1.

In comparison 1

Hydrogen peroxide production: As shown in Figure 7E, significantly increased levels of hydrogen peroxide were found in mutant A β PP cells (P=0.004) relative to untransfected N2a cells.

Lipid peroxidation: Significantly increased levels of lipid peroxidation were found in mutant A β PP cells (P=0.002), but lipid peroxidation levels were unchanged in mutant A β PP cells treated with SS31, Mdivi1 and in combination of SS31+Mdivi1 (Figure 7F).

Cytochrome c oxidase activity: As shown in Figure 7G, significantly reduced levels of cytochrome c oxidase activity were found in mutant A β PP cells (P=0.001), but cytochrome c oxidase activity levels were unchanged in mutant A β PP cells treated with SS31, Mdivi1 and in combination of SS31+Mdivi1.

ATP production: Significantly reduced levels of ATP production were found in mutant A β PP cells (P=0.002), but ATP production levels were unchanged in mutant A β PP cells treated with SS31, Mdivi1 and in combination of SS31+Mdivi1 (Figure 7H).

GTPase Drp1 activity: Significantly higher levels of GTPase Drp1 activity were found in N2a cells transfected with mutant A β PP cDNA (P=0.04) relative to untransfected N2a cells (Figure 8B). GTPase Drp1 activity was reduced in Mdivi1 and SS31+Mdivi1 treated mutant A β PP cells, but not significant.

Comparison 2

Hydrogen peroxide production: In comparison 2 - as shown in Figure 7I, significantly reduced levels of hydrogen peroxide were found in SS31 ($P=0.004$), Mdivi1 ($P=0.03$) and SS31+Mdivi1 treated mutant A β PP cells relative to untreated mutant A β PP cells ($P=0.004$).

Lipid peroxidation: In comparison 2 - Significantly reduced levels of lipid peroxidation were found in SS31 ($P=0.004$), Mdivi1 ($P=0.003$) and SS31+Mdivi1 treated mutant A β PP cells relative to untreated mutant A β PP cells ($P=0.002$) Figure 7J).

Cytochrome c oxidase activity: As shown in Figure 7K, significantly increased levels of cytochrome c oxidase activity were found in SS31 ($P=0.002$), Mdivi1 ($P=0.03$) and SS31+Mdivi1 ($P=0.001$) relative to untreated mutant A β PP cells.

ATP production: Significantly increased levels of ATP production were found in SS31 ($P=0.01$), Mdivi1 ($P=0.01$) and SS31+Mdivi1-treated mutant A β PP cells ($P=0.005$) relative to untreated mutant A β PP cells (Figure 7L).

GTPase Drp1 activity: Significantly decreased levels of GTPase Drp1 activity in mutant A β PP cells treated with SS31 ($P=0.04$), Mdivi1 ($P=0.01$) and combination treatment of SS31+Mdivi1 ($P=0.004$) relative to untreated mutant A β PP cells (Figure 8C). These observations suggest that mutant A β PP increases GTPase Drp1 activity – on the other hand SS31, Mdivi1 and combination of SS31+Mdivi1 significantly decreases GTPase Drp1 activity. However, the combination treatment of SS31+Mdivi1 showed the higher reduction of GTPase Drp1 activity than individual treatments of SS31 and Mdivi1.

Discussion

The purpose our study was to determine the synergistic protective effects of mitochondria-targeted antioxidant SS31 and mitochondria division inhibitor 1 in Alzheimer's disease. Our recent studies revealed that cell permeable mitochondria-targeted antioxidant SS31 is protective against amyloid beta induced synaptic and mitochondrial toxicities in both cell and mouse models of AD [15, 52]. Further, our *in vitro* studies revealed that Mdivi1 is protective against A β -induced excessive mitochondrial fragmentation and mitochondrial dysfunction in AD neurons [47]. Several recent mouse model studies of AD, also revealed that Mdivi1 is protective against A β -induced synaptic and mitochondrial toxicities, particularly Mdivi1 reduces excessive mitochondrial fragmentation [53–54]. Very little is known about synergistic protective effects of SS31 and Mdivi1 in AD neurons. Therefore, in the present study, we sought to determine the synergistic protective effects of SS31 and Mdivi1 against A β -induced toxicities in AD. We also assessed protective effects of SS31, Mdivi1 individually and in combination in mouse neuroblastoma cells. We transfected N2a cells with mutant A β PP cDNA (Swe/Ind) and transfected cells further treated with SS31, Mdivi1 and combination of SS31 and Mdivi1. Using biochemical methods, we assessed mitochondrial function by measuring hydrogen peroxide, lipid peroxidation, cytochrome c oxidase activity and mitochondrial ATP. Using biochemical methods, we also measured GTPase Drp1 activity, cell survival and apoptotic cell death. Amyloid beta levels were

measured using sandwich ELISA and using real-time quantitative PCR, we assessed mtDNA to nDNA ratios in all groups of cells.

Mutant A β PP cDNA transfections were successful as evidenced by the presence of 110 kDa mutant full-length A β PP and A β 40 and A β 42 in N2a cells transfected with mutant A β PP cDNA. We found significantly reduced levels of A β 40 and A β 42 in mutant A β PP cells treated with SS31, Mdivi1 and SS31+Mdivi1 and A β 42 levels were reduced much higher in SS31+Mdivi1 treated cells than individual treatments of SS31 and Mdivi1.

MtDNA copy number is increased in N2a cells treated with SS31, Mdivi1 and SS31+Mdivi1. It is interesting to observe that mtDNA copy number is significantly increased in mutant A β PP cells treated with SS31, Mdivi1 and SS31+Mdivi1 relative to untreated mutant A β PP cells; however, mtDNA copy number is increased much higher in SS31+Mdivi1 treated cells than individual treatments of SS31 and Mdivi1. Cell survival rate is higher in all treatments. Mitochondrial function is improved in N2a cells treated with SS31, Mdivi1 and SS31+Mdivi1. It is noteworthy to observe that combination treatment improved mitochondrial function much higher than individual treatments. Mitochondrial dysfunction was reduced in mutant A β PP cells treated with SS31, Mdivi1 and SS31+Mdivi1. Similarly, GTPase Drp1 activity is reduced in mutant A β PP cells treated with SS31, Mdivi1 and SS31+Mdivi1, however, reduced levels of GTPase Drp1 activity is higher in mutant A β PP cells treated with SS31+Mdivi1 than individual treatments. These observations strongly suggest that combined treatment of SS31+Mdivi1 is effective than individual treatments of SS31 and Mdivi1 against A β -induced mitochondrial toxicities in AD. Therefore, we propose that combination treatment of SS31 and Mdivi1 is a better therapeutic strategy to reduce mitochondrial and A β -induced toxicities for AD. Ours is the first study to investigate combined treatment of mitochondria-targeted antioxidant SS31 and mitochondrial division inhibitor in AD neurons.

Amyloid beta levels

Increasing evidence suggests that A β production and accumulation increases with disease progression in AD patients and A β PP mouse models [55–58]. Several recent studies also reported that mitochondria-targeted molecules and mitochondrial division inhibitors reduce A β levels and mitochondrial and synaptic toxicities in AD neurons [47,53,54]. However, there are no published reports that investigated using combined treatments of mitochondria-targeted molecules and mitochondrial division inhibitors. Our current study is the first to investigate combined treatment of mitochondria-targeted molecules and mitochondrial division inhibitors. As described above, we found significantly lower levels of A β 40 levels in the mutant A β PP cells treated with SS31 and Mdivi1 and in combination treatment of Mdivi1+SS31 compared to untreated mutant A β PP cells (Figure 3A). Not surprisingly, we also found significantly lower levels of A β 42 levels in the mutant A β PP cells treated with SS31 (P=0.004) and with Mdivi1 (P=0.03) and in combination treatment of Mdivi1+SS31 (P=0.002) compared to mutant A β PP cells (Figure 3B). It is interesting to note that combination treatment reduced A β 42 levels much higher than individual treatments of Mdivi1 and SS31 in mutant A β PP cells. Overall, these observations revealed that a combination treatment is effective than individual treatments.

Mechanistically, the observed higher reduced levels of A β 42 in combined treatment may be due to 1) reduced free radicals in the mitochondria with SS31 treatment and 2) reduced excessive mitochondrial fragmentation with Mdivi1 treatment, the synergistic effect of combined treatment is protective to mitochondria by scavenging mitochondria-generated free radicals and decreasing fission activity and allowing mitochondria to perform their function normally. To confirm these notions, further research is needed using multiple lines of A β PP mouse models.

mtDNA copy number and mitochondrial function

Mitochondrial copy number is a strong indicator of health of mitochondria. In healthy state, the quality of mitochondria is good – with 1) normal mitochondrial biogenesis (higher expressions of mitochondrial biogenesis genes, including PGC1alpha, Nrf1, Nrf2 and TFAM), 2) normal mitochondrial function, 3) mitochondrial trafficking and 4) higher mtDNA copy number, whereas in disease state such as AD, quality of mitochondria is bad with 1) defective mitochondrial biogenesis (meaning with reduced expressions of PGC1alpha, Nrf1, Nrf2 and TFAM genes), 2) defective mitochondrial function and 3) lower mtDNA copy number and 4) defective mitochondrial trafficking. These abnormal mitochondria features have been rescued with mitochondria-targeted molecules such as SS31 [22,24] and MitoQ [22] and mitochondrial division inhibitors [47,53,54]. However, there are no published reports that investigated combined treatment of both mitochondria-targeted molecules and mitochondrial division inhibitors in AD and other neurological diseases.

Ours the first study that investigated the synergistic protective effects of mitochondria-targeted molecules and mitochondrial division inhibitors in AD neurons. As described above, we found increased mtDNA copy number and reduced mitochondrial dysfunction in combined treatment of SS31 and Mdivi1 in mutant A β PP cells much higher than individual treatments. Our study is the step one in a positive direction – we still need to study mRNA and protein levels of mitochondrial biogenesis genes, mitochondrial trafficking and synaptic properties of AD neurons in AD cells and AD mouse models.

Cell survival and apoptotic cell death

It is well established that mutant A β PP and A β causes lower cell survival and higher apoptotic in AD neurons [59]. In the current study, we found mutant A β PP cells showed reduced levels of cell survival and increased levels apoptotic cell death in mutant A β PP cells – on the other hand, mitochondria-targeted molecule SS31 and mitochondrial division inhibitor 1 enhanced cell survival and reduced apoptotic cell death in mutant A β PP cells - this effect is much stronger in combined treatment of SS31 and Mdivi1 in mutant A β PP cells, strongly suggest that combined treatment is protective against A β - and mitochondria-induced cell death in AD neurons.

In summary, we cautiously say that combined treatment of mitochondria-targeted antioxidants and mitochondrial division inhibitors are better therapeutic approaches for AD and other neurological diseases.

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Abbreviations

Mdivi1	Mitochondrial division inhibitor 1
SS31	Szeto Schiller 31
Aβ	amyloid beta
AβPP	amyloid beta precursor protein
nDNA	nuclear DNA
mtDNA	mitochondrial DNA

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Figure 1.

Experimental design of synergistic protective effects of mitochondria-targeted antioxidant SS31 and mitochondria division inhibitor 1 in Alzheimer's disease.

1. Untransfected N2a cells
2. N2a cells transfected with mAPP cDNA

Figure 2. Immunoblotting analysis of mutant A β PP cells

Mouse neuroblastoma (N2a) cells were transfected with mutant A β PP cDNA and immunoblotting analysis was conducted using protein lysates from transfected and untransfected N2a cells with mutant A β PP cDNA. A protein band of 110kDA full-length A β PP was found in N2a cells transfected with mutant A β PP cDNA.

Figure 3. Amyloid beta levels

Using Sandwich ELISA, we measured Aβ40 and Aβ42 levels in mutant AβPP cells – 1) mutant AβPP treated with SS31, 2) mutant AβPP cells treated with Mdivi1 and 3) mutant AβPP cells treated with both SS31 and Mdivi1. A. Significantly lower levels of Aβ40 levels were found in the mutant AβPP cells treated with SS31 (P=0.001) and with Mdivi1 (P=0.003) and in combination treatment of Mdivi1+SS31 (P=0.004) compared to untreated mutant AβPP cells. B. Significantly reduced levels of Aβ42 levels were found in the mutant AβPP cells treated with SS31 (P=0.004) and with Mdivi1 (P=0.03) and in combination treatment of Mdivi1+SS31 (P=0.002) compared to untreated mutant AβPP cells.

Figure 4. Mitochondrial DNA copy number

Using NovaQuant Kit, we measured DNA copy numbers of 2 nuclear genes and 2 mitochondrial genes. **A.** Significantly increased mtDNA copy number/cell was found N2a cells treated with SS31 (P=0.01), Mdivi1 (P=0.03) and in combination treatment of SS31+Mdivi1 (P=0.002). **B.** We found significantly decreased mtDNA copy number in mutant A β PP cells relative to untransfected N2a cells with mutant cDNA. MtDNA copy number is increased in mutant A β PP cells treated with SS31, Mdivi1 and SS31+Mdivi1, but not significant. **C.** We found mtDNA copy number is significantly increased in mutant A β PP cells treated SS31 (P=0.01), Mdivi1 (P=0.01) and SS31+Mdivi1 (P=0.005) (Figure 3B) relative to mutant A β PP cells. However, the increased levels of mtDNA copy number is much higher for combination treatment than individual treatments of SS31 and Mdivi1.

Figure 5. Immunostaining analysis of TOM20

Immunostaining analysis was performed using TOM20 antibody, in order to determine mitochondrial network in mutant A β PP cells treated with SS31, Mdivi1 and SS31+Mdivi1. Mitochondrial network was significantly increased in mutant A β PP cells treated with SS31 (P=0.01), Mdivi1 (P=0.02) and SS31+Mdivi1 (P=0.004). Statistical significance was higher in SS31+Mdivi1 treated mutant A β PP cells than individual treatments of SS31 and Mdivi1.

Figure 6. Cell survival and apoptotic cell death

To determine the effect of SS31, Mdivi1 and Mdivi1 treatment of N2a cells, we assessed cell survival and apoptotic cell death. **A.** Significantly increased cell survival in SS31 ($P=0.02$), Mdivi1 ($P=0.02$) and SS31+Mdivi1 treated N2a cells ($P=0.004$) relative to untreated N2a cells. **B.** Significantly reduced apoptotic cell death was observed in SS31 ($P=0.002$), Mdivi1 ($P=0.002$) and SS31+Mdivi1 treated N2a cells ($P=0.001$) relative to untreated N2a cells. **C.** Significantly reduced cell survival was found in mutant A β PP cells relative to untransfected N2a cells ($P=0.005$). **D.** Significantly increased levels of cell survival were found in mutant A β PP cells treated with SS31 ($P=0.004$), Mdivi1 ($P=0.002$) and SS31+Mdivi1 ($P=0.001$). **E.** Significantly increased apoptotic cell death levels found in mutant A β PP cells relative to untransfected N2a cells ($P=0.001$). On the other hand, the levels of apoptotic cell death were significantly decreased in mutant A β PP cells treated with SS31 ($P=0.003$), Mdivi1 ($P=0.04$) and SS31+Mdivi1 ($P=0.001$) relative to untreated mutant A β PP cells. **F.** Significantly decreased levels of apoptotic cell death were found in mutant cells treated SS31 ($P=0.002$), Mdivi1 ($P=0.001$) and SS31+Mdivi1 ($P=0.0001$) relative to untreated mutant APP cells.

Figure 7. Mitochondrial function

The parameters included hydrogen peroxide production, cytochrome c oxidase activity, lipid peroxidation (4-hydroxy-nonenal) and ATP production were assessed in 1). N2a cells and 2) mutant APP cells treated and untreated with SS31, Mdivi1 and SS31+Mdivi1. **A.** Hydrogen peroxide production: Significantly reduced levels of hydrogen peroxide were found in SS31 (P=0.01), Mdivi1 (P=0.03) and SS31+Mdivi1 (P=0.004) treated N2a cells relative to untreated N2a cells. **B.** Lipid peroxidation: Significantly reduced levels of lipid peroxidation were found in SS31 (P=0.03), Mdivi1 (P=0.04) and SS31+Mdivi1 (P=0.01) treated N2a cells relative to untreated N2a cells. **C.** Cytochrome c oxidase activity: Significantly increased levels of cytochrome c oxidase were found in SS31 (P=0.02), Mdivi1 (P=0.03) and SS31+Mdivi1 (P=0.004) treated N2a cells relative to untreated N2a cells. **D.** ATP production: Significantly increased levels of ATP were found in SS31 (P=0.02), Mdivi1 (P=0.02) and SS31+Mdivi1 (P=0.004) treated N2a cells relative to untreated N2a cells. **E.** Hydrogen peroxide production: Significantly increased levels of hydrogen peroxide were found in mutant AβPP cells (P=0.004) relative to untransfected N2a cells. **F.** Lipid peroxidation: Significantly increased levels of lipid peroxidation were found in mutant AβPP cells (P=0.002), relative to untransfected N2a cells. **G.** Cytochrome c oxidase activity: Significantly reduced levels of cytochrome c oxidase activity were found in mutant AβPP cells (P=0.001) relative to untransfected N2a cells. **H.** ATP production: Significantly reduced levels of ATP production were found in mutant AβPP cells (P=0.002) relative to untransfected N2a cells. **I.** Significantly reduced levels of hydrogen peroxide were found in SS31 (P=0.004), Mdivi1 (P=0.03) and SS31+Mdivi1 treated mutant AβPP cells relative to mutant AβPP cells (P=0.004). **J.** Lipid peroxidation: In comparison 2 - Significantly reduced levels of lipid peroxidation were found in SS31 (P=0.004), Mdivi1 (P=0.003) and SS31+Mdivi1 treated mutant AβPP cells relative to mutant AβPP cells (P=0.002). **K.** Significantly increased levels of cytochrome c oxidase activity were found in SS31 (P=0.002), Mdivi1 (P=0.03) and SS31+Mdivi1 (P=0.001) treated mutant AβPP cells relative to untreated mutant AβPP cells. **L.** ATP production: Significantly increased levels of ATP production were found in SS31 (P=0.01), Mdivi1 (P=0.01) and SS31+Mdivi1 treated mutant AβPP cells (P=0.005) relative to untreated mutant AβPP cells.

Figure 8. GTPase Drp1 activity

GTPase Drp1 activity was assessed in N2a cells treated with SS31, Mdivi1 and combined treatment of SS31+Mdivi1 relative to untreated N2a cells. Significantly decreased GTPase Drp1 activity was found in SS31-treated N2a cells ($P=0.02$), Mdivi1 treated N2a cells ($P=0.03$) and SS31+Mdivi1 treated N2a cells ($P=0.01$). GTPase Drp1 activity was also assessed in mutant APP cells treated and untreated with SS31, Mdivi1 and SS31+Mdivi1. Data were compared the data 2 ways – in comparison 1– untransfected N2a cells with mutant APP cDNA with the treatments SS31, Mdivi1, and SS31+Mdivi1, and in comparison 2– mutant A β PP cDNA transfected N2a cells (mutant A β PP cells) with the treatments of SS31, Mdivi1 and SS31+Mdivi1. In comparison 1, significantly higher levels of GTPase Drp1 activity were found in N2a cells transfected with mutant A β PP cDNA ($P=0.04$) relative to untransfected N2a cells (Figure 8B). GTPase Drp1 activity was reduced in Mdivi1 and SS31+Mdivi1 treated mutant A β PP cells, but not significant. In comparison 2, significantly decreased levels of GTPase Drp1 activity in mutant A β PP cells treated with SS31 ($P=0.04$), Mdivi1 ($P=0.01$) and combination treatment of SS31+Mdivi1 ($P=0.004$) relative to untreated mutant A β PP cells (Figure 8C).