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Exploring neuroprotective effect of a combined strategy of LM11A-31, Resveratrol, and Methylene Blue as a treatment for Alzheimer's Disease pathology: A pilot study

Thesis in Neuroscience

By

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Abstract:

Alzheimer's Disease (AD) is the most common form of dementia and neurodegenerative disorder the leads to cognitive impairment such as severe memory loss. AD is rapidly increasing amongst the human population with no treatment available. There are many drugs currently being looked at, but none are able to treat to the sporadic form of the disease. AD patients experience brain dystrophy thought to be induced by molecular hallmarks of the disease such as Aβ accumulation, hyperphosphorylated tau, oxidative stress and neuroinflammation. Some drugs that are being looked at as possible treatments for AD are LM11A-31, Resveratrol, Methylene Blue. Each of these drugs are thought to exhibit neuroprotective effects but is unclear how they promote cell survivability in AD models and if this neuroprotective effect is transferable from rat models to human patients. Also, the neuroprotective effect demonstrated in past studies is fairly limited. As LM11A-31, Resveratrol, and Methylene Blue are believed to modulate similar molecular pathways attributed causing neuronal dystrophy in AD patients, I present a pilot study looking at if a combined treatment strategy of all three drugs will increase neuroprotection against a common neurotoxin when used in excess, NMDA, in an in vitro model system. Neuronal cultures were stressed with individual compounds and combined treatments of NMDA, LM11A-31, Resveratrol, and Methylene Blue. I report LM11A-31, Resveratrol, and Methylene Blue did not offer strong neuroprotective properties against NMDA individually or in two combinations of the drugs. Limited neurotoxicity was also reported when treating cells with either FAB or NMDA.

Table of Contents

Introduction:

Alzheimer's Disease Hallmarks and Neuronal death pathways

Alzheimer's disease (AD) is a form of dementia that causes progressive loss of cognitive function including impaired memory recollection and formation. The neurodegenerative nature of the disease has been shown to cause atrophy in regions of the brain. MRI scans of Alzheimer's disease patients have indicated that a decrease in gray matter (cell body or soma of the neuron) volume when compared to MRI scans of healthy participants (Karas et al. 2004). A significant amount of brain atrophy illustrated in Alzheimer's disease patients is related to the cognitive impaired behavior exhibited by such patients. Common symptoms of Alzheimer's disease include short- and long-term memory loss(hallucinations, difficulty performing tasks, poor judgement, and behavior changes such as increase in anxiety and depression (Alzheimer's Association 2016). AD is the most common form of dementia with approximately 46.8 million cases around the world and increasing every year with no cure available (Grabher 2018). AD not just only affects the patients themselves but their family and care givers around them. As the patients age, symptoms tend to worsen to the point in which they cannot fully function by themselves. There are two types of AD: sporadic AD and familiar/early onset AD. Sporadic AD, which is the most common form, patients are diagnosed at age 65 or older. Familial/early onset form which is typically genetically linked and diagnosed in patients younger than 64. Regardless of which type of AD a patient is diagnosed with, patients tend to live with the disease for years after diagnosis increasing their neuronal dystrophy and cognitive impairment as they age. This puts financial, emotional, and physical stress to themselves along with their declining cognitive abilities as well as on their family members and caregivers. The reported total cost of care for all AD patients in the United States was $305 billion and on average $25,215 per patient (Wong

2020). Without a substantial treatment for AD cases and AD's detrimental effects to the patients and their families will continue to increase exponentially.

There are currently very few if any possible treatments for Alzheimer's Disease due to the complex nature of the illness. Alzheimer's Disease was first seen by Dr. Alois Alzheimer in 1911 in his patient Auguste Deter although he did not know at the time what her symptoms of memory loss and difficulty with tasks were caused by at the time (Bondi et al. 2017). Alzheimer drew sketches of AD pathology hallmarks known today as Aβ plaques and tau tangles, but it was not until 1968 that the cellular pathology of AD was linked to low cognitive performance on standardized tests, correlating the neuronal dystrophy and cognitive impairment symptoms of AD. Until 1976 AD was considered a rare disease as for the majority, only patients younger than 65 were diagnosed with presenile AD. Due to the relatively recent acceptance of AD as a leading cause of death in the elderly, research has been limited which directly caused difficulty in not only diagnosing AD but finding an effective treatment. As of 2019, the current failure rate of AD drug therapies on the market remains at 99% failure rate (Cummings et al 2019). Understanding the pathological development and having the time to do is crucial to eventually discovering a cure.

Two theorized neuropathological causes of Alzheimer's disease symptoms are the production of Aβ plaques, and oxidative stress. Aβ plaques in Alzheimer's Disease are primarily formed from $A\beta_{1\text{-}42}$ peptides (Takahashi et al. 2017). The peptides are created when amyloid precursor protein (APP) is cleaved by β-secretase and γ-secretase. APP can also be cleaved by cleaved by α-secretase and does not result in the formation of $A\beta_{1\text{-}42}$ peptides. Cleaving APP at the beta site leads to the production of amyloid-beta and it is the excessive accumulation of Aβ that leads to neuronal loss and eventually cognitive impairment. An excess of Aβ produces Aβ

oligomers that cause toxicity within the cells by potentially affecting many different parts of the neurons. The aggregation of Aβ has been linked to the production of oxidative stress by the coordination of reactive oxygen species (ROS) and metal ions such as copper, iron, and zinc (Cheignon et al. 2018). The presence of the redox metal ions plays a role in the aggregation of Aβ plaques and increases ROS inducing oxidative stress and cellular toxicity. Aβ induced oxidative stress can also be linked to mitochondria dysfunction which can result in apoptosis of the cell (Rajasekhar et al. 2015). An antioxidant may prevent further oxidative stress damage from Aβ therefore utilizing Aβ as a stressor would be a logical strategy. The toxicity of Aβ oligomers can affect more than just oxidative stress levels in cells, for instance synaptic dysfunction may be induced by Aβ oligomers. The Aβ oligomers can bind to receptors such as the NMDA, glutamate, and AMPA receptors limiting neurotransmission and synaptic communication. When neurons are unable to effectively communicate the cell can be severely damaged and as a result cell viability decrease (Rajasekar et al. 2015)/

Tau is a neuronal protein also thought to be implicated abnormally in AD patients making it another hallmark of AD pathology. The phosphorylation of Tau plays a key role in maintaining microtubule stability and axonal transport (Johnson and Stoothoff 2004). When tau is hyperphosphorylated it can lead to the production of neuronal tangles impacting neuronal function and cell survivability. The proper regulation of tau phosphorylation is essential to maintain microtubules stability, integrity of the cells and the overall survivability of neurons. Improper phosphorylation of tau increases neuronal dystrophy and therefore leads to the behavioral issues associated with AD patients. Kinases that have been associated with regulating tau phosphorylation are glycogen synthase kinase 3β (GSK3β) and cyclin-dependent kinase 5 (Cdk5, Johnson and Stoothoff 2004). Over expression or phosphorylation of GSK3β and Cdk5 has been

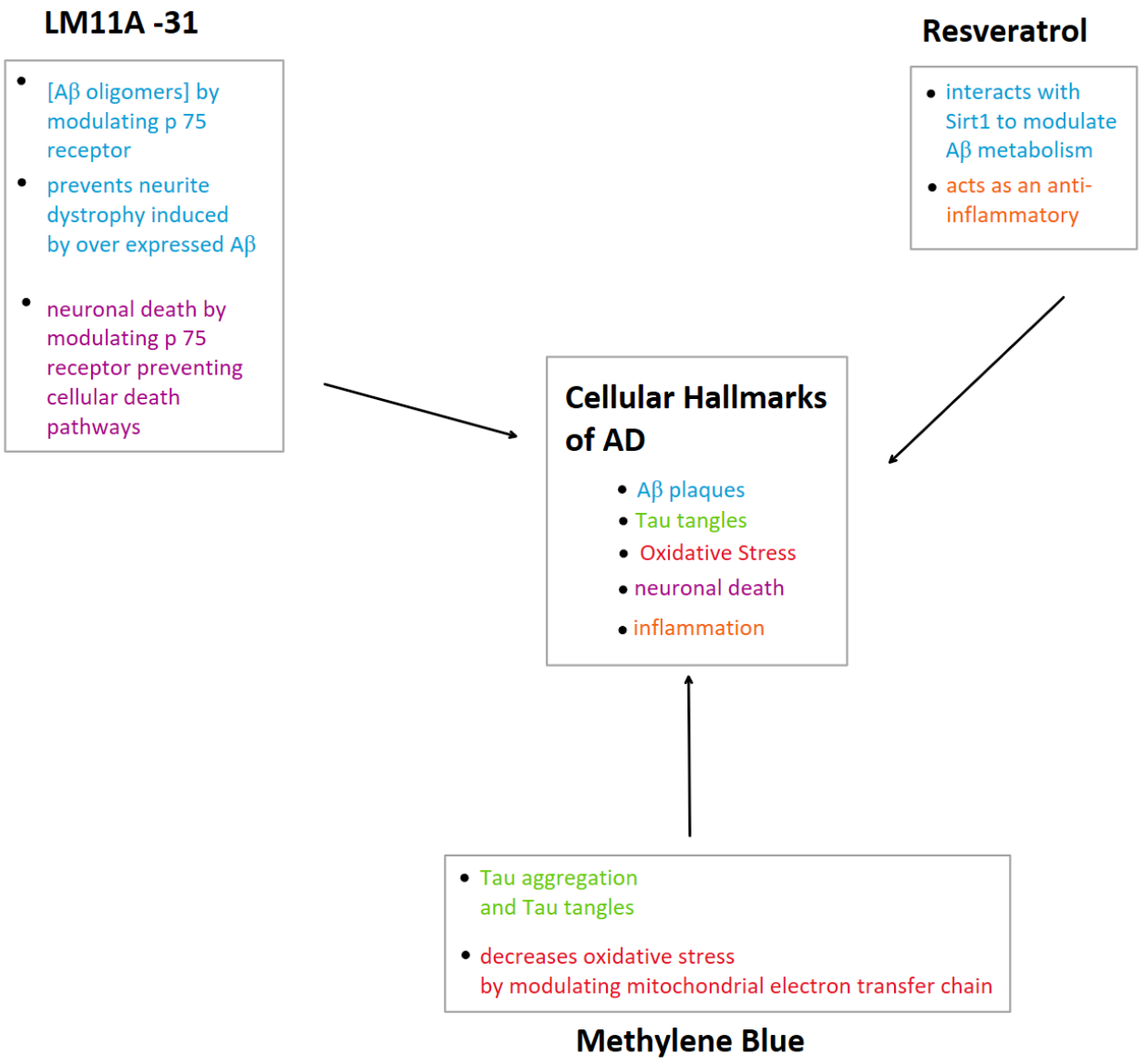
linked to the increase of the hyperphosphorylation of tau that eventually produces the NFT found in AD. GSK3β and Cdk5 may directly regulate tau phosphorylation or because these kinases regulate other various proteins, they may play a role in pathways associated with tau phosphorylation and then cause a downstream effect leading to the development of NFT. A therapeutic technique might be to the treat parts, if not a whole, pathway leading to tau phosphorylation to limit the production of NFT and cognitive impairment the results from disrupted microtubule stability and neuronal death in AD.

Another hallmark cellular response of Alzheimer's disease is neuroinflammation which may be presented prior to the formation of Aβ plaques for it remains inconclusive which in the cell occurs first and causes the other. However, increased amounts of Aβ trigger can be degraded by microglia producing a proinflammatory and anti-inflammatory response (Heneka et al. 2015). When the microglia are activated to degrade soluble Aβ the proinflammatory response comes from the cytokine's tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1,6,12 and18. The cytokines IL-4,10,13 enact an anti-inflammatory response. While the immune response of microglia appears to have a beneficial effect by degrading Aβ, an increase in TNF α - and IL-1β has been related to a decrease in synaptic function and production of ROS. Since neuroinflammation is present in almost all of Alzheimer Disease patients, a drug that exhibits an anti-inflammatory may be useful in treating the disease.

While these hallmarks of Alzheimer's disease are present in patients suffering with the disease, it is unclear if they are responsible for the development of the disease or a byproduct of a separate pathway. Popular theories suggest Aβ accumulations and hyperphosphorylated tau have shown neurotoxic effects by inducing oxidative stress leading to a loss in cell viability and increase in inflammation (Takahashi et al. 2017). But it is also possible that the presence of ROS and

inflammation leads to a production of Aβ plaques and NFT. What is apparent is a significant decrease in cell survivability in patients with Alzheimer's disease. The current 99% failure rate of drugs on the marker to treat AD suggests it is not a singular compound that will serve as an effective treatment for AD, but a combination. Singular treatments have been done and they are not working potentially due to multiple hallmarks of AD being left untreated. Due to the complexity of AD, it is unlikely that a single drug will be able to target Aβ plaque and tau tangle formation as well as keeping inflammation and oxidative stress at bay.

While the pathology of the hallmarks of AD are connected to one another in some capacity, there are proteins and pathways independent to each hallmark limiting a singular compound treatment's effectiveness. Three drugs that have independently shown some, though limited, protection against the hallmarks of AD are LM11A-31, Resveratrol, and Methylene Blue. Below is a schematic outline of the hallmarks of AD each of the drugs has been shown to target(schematic 1). Mutliple hallmarks and pathways are implicated as a targets by each drug and they may target the same hallmark as another drug. LM11A-31 and Resveratrol are thought to effect the accumilation of Aβ plaques. There is some evidence that LM11A-31 and Resveratrol target oxdative stress pathways as does Methylene Blue, however, oxidative stress is considered a by product/downstream effect and less so of a major target like Aβ accumulation.



Schematic 1: Alzheimer's Disease hallmarks potentially impacted by LM11A-31, Resveratrol and/or Methylene Blue. Research has been done on each of the compounds focusing on common hallmarks of AD and multiple compounds treat are thought to target more than one hallmark and hallmarks treated by the other two compounds.

Neurotrophins

A key factor to evaluating LM11A-31 neuroprotective ability against the hallmarks of Alzheimer's Disease is by understanding the crucial molecules of neuronal survival, neurotrophins. Neurotrophins are essential ligands that mediate neuronal survival. There are many types of neurotrophins in including nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and neurotrophin 3 and 4 (NT3, NT4). Neurotrophin levels have been known to decrease in patients with AD, some suggesting that BDNF levels be used as a biomarker for AD (Tanila 2017). In one study NGF gene transfers were done in AD patients to observe if injected NGF gene therapy would be able to prevent cholinergic neurodegeneration (Tuszynski et al. 2015). The researcher reported an increase in Cholinergic axonal sprouting when labeling grafts of each patient for the p75 receptor found on cholinergic neurons in the basal forebrain. This would suggest an increase in neuronal communication and potentially a neuroprotectant effect. The researchers did see record of one patient surviving 10 years after the NGF gene transfer, but out of 8 patients available for the study there were multiple that died within 3-5 years and one within 3 months of the procedural. The patients were an array of ages when the procedure was done, most between the age 70-73, while 56- and 78-year-old patients were at the extreme ends of the spectrum. It is possible that the patients passed for other reasons than any possible adverse effects from the procedure, but it is still questionable whether a gene transfer is the safest and most effective method to address neurotrophin decrease in AD patients. Targeting a neurotrophic receptor without injection to the brain may be a safer alternative to increase neurotrophin levels.

Each of these neutrophins have individual receptors that they can bind to called tropomyosin receptor kinase (TRK) A, B, and C (Longo and Massa 2013). NGF, BDNF, NT3 and NT4 can also all bind to the p75 receptor. The p75 receptor ($p75^{NTR}$) is a type of tumor

necrosis factor receptor which has multiple binding sites for different ligands. $p75^{NTR}$ is involved in a couple different pathways regarding neuronal phosphoinositide 3-kinase (PI3K)–AKT pathway, mitogen-activated protein kinase (MAPK), and AMP–protein kinase A (PKA) pathway (Longo and Massa 2013). Such pathways are theorized to be affected in Alzheimer Disease patients making the $p75^{NTR}$ a potential target. While neurotrophins binding to their respective Trk receptor tend to stimulate neuronal growth and survival, neutrophins binding to the p75 receptor can promote either neuronal death or survival. When precursor neutrophins bind to a protein called sortilin (SORT 1) and the $p75^{NTR}$ the cell is more likely to induce apoptosis. Pro-neurotrophin NGF (pro-NGF) which is the neurotrophin NGF with an N-terminal that is typically cleaved off before binding to $p75^{NTR}$ with the sortilin protein (Massa et al. 2006). Neurotrophin with the N-terminals cleaved off are considered mature neurotrophins. The combination of proNGF and sortilin binding to the $p75^{NTR}$ are believed to be why binding to the $p75^{NTR}$ does not always promote cellular survivability. Researchers suggest that the complex consisting of pro-neurotrophins and sortilin binding to the $p75^{NTR}$ results from an interaction between SORT 1 and an extracellular juxta membrane of the $p75^{NTR}$ and SORT 1 cleaving an intracellular domain of the $p75^{NTR}$ (Skeldal et al. 2021). One treatment strategy to prevent the pro-neurotrophin and SORT1 complex from inducing apoptosis via binding to the $p75^{NTR}$ would be to limit the functionality of the p75. Another molecule that has a higher affinity for the $p75^{NTR}$ than the SORT1/pro-neurotrophin complex and can modulate the $p75^{NTR}$ without making it completely in active would be ideal. Blocking complete neutrophins from binding to the $p75^{NTR}$ theoretically should allow more neutrophins to bind to their respective Trk receptor instead of $p75^{NTR}$, avoiding apoptosis caused by the SORT1/pro-neurotrophin complex and cleaving of $p75^{NTR}$.

P75 receptor and LM11A-31

Besides the SORT1/pro-neurotrophin complex, amyloid beta(Aβ) has also been shown to bind to $p75^{NTR}$ causing neuronal death. In primary cells cultured from transgenic mice with the $p75^{NTR}$ deleted and an AD mouse model (Thy1-hAPPLond/Swep75^{NTR}) there was a decrease in neuronal dystrophy in cells from both the hippocampus and basal forebrain region (Knowles et al. 2009). Compared to the mice that expressed the $p75^{NTR}$ ($p75^{NTR+/+}$), the mice that had the $p75^{NTR}$ deleted ($p75^{NTR-/-}$) there were noticeably lower concentrations of Aβ oligomers present. This suggests that the $p75^{NTR}$ plays a crucial role in modulating the presence of Aβ plaques and therefore the development of Alzheimer's Disease making $p75^{NTR}$ valuable target for treating Alzheimer's Disease. However, the results obtained in this study is only relevant to an organism that has the $p75^{NTR}$ removed. While it is useful to study how a deleted gene would affect an organism to better understand the role $p75^{NTR}$ plays in the neuronal survival, the deletion of the $p75^{NTR}$ is not a possible treatment for Alzheimer's Disease patients. Also, the researchers in this study only looked at the concentration of Aβ compared to the neuronal survivability in the $p75^{NTR+/+}$ and $p75^{NTR-/-}$ mice. It is reasonable to consider that the cell survivability was not affected just by the concentration of Aβ developed by the AD mouse model but also by any of the pathways the $p75^{NTR}$ plays a role in and other ligands that bind to $p75^{NTR}$. Model systems that involve the modulation of the $p75^{NTR}$, rather a deletion of $p75^{NTR}$, would provide a more useful strategy in treating Alzheimer's Disease.

The LM11A compounds that have exhibited neuronal protectant effects on cultured embryonic day 16/17 hippocampus neurons are LM11A-24 and LM11A-31(Massa et al. 2006). These compounds were found to promote neuronal survival by their binding to NGF and the

$p75^{NTR}$ causing an increase in survivability. Due to the TRK A receptor (NGF's TRK receptor) being less abundant than TRK B (BDNF's TRK receptors) in the hippocampus NGF should not be promoting neuronal survival through the TRK A receptor. The researchers evaluated neuronal survivability in hippocampal cells when exposed to LMA11 compounds and exogenous BDNF and NGF. Cells exposed to BDNF but not treated with LMA11 compounds had a high level of cell survivability. When the LM11A compounds were added to the cells exposed to BDNF, the percentage of survivability decreased a bit, most dramatically in when LM11A-31 was added. This is not preferable as a decrease in hippocampal cells will only worsen AD symptom of memory loss instead of treating AD, but LM11A-31 and LM11A-24 were able to keep the percent survivability at a reasonably high level. On the other hand, cells treated with exogenous NGF and the LM11A compounds showed an increase in survivability compared to cells just treated with NGF. As there are very few if any TRK A receptors in the hippocampus cells cultured this would indicate that the increase of cell survivability was due to an interaction between the $p75^{NTR}$ and NGF. This suggests that LM11A-31 and LM11A-24 structurally may interact less with BDNF than NGF and therefore allows for more binding to the $p75^{NTR}$ increasing cell survival in hippocampal neurons. While the addition of LM11A compounds seemed to decrease cell survival in the cells treated with BDNF instead of increasing it when exposed to NGF, the compounds LM11A-31 and LM11A-24 were able to mediate these toxic effects. Also, the cells were treated with exogenous neurotrophins, perhaps if the levels of BDNF were at a more natural concentration, more BDNF would just bind to TRKB instead of the LM11A compounds and allow for neuronal survival. The next matter to consider is if LM11A compounds that bind to NGF and $p75^{NTR}$ and promote cellular survivability, why does proNGF

binging to $p75^{NTR}$ activate neuronal death pathways and can the LM11A compounds battle this toxicity?

The LM11A compounds, LM11A-31 and LM11A-24 were found to prevent cellular death that is mediated by proNGF (Massa et al. 2006). Due to oligodendrocytes not expressing the TRK A receptor but do express $p75^{NTR}$, NGF and proNGF promote cellular death in oligodendrocytes. With the addition of LM11A compounds cellular death was not increased in oligodendrocytes treated with NGF. Also, the cellular death was inhibited in cells treated with LM11A compounds and proNGF. This indicates that targeting the $p75^{NTR}$ with LM11A compounds, specifically LM11A-31 and LM11A-24, has potential to be therapeutic target for multiple cell types, not just neurons. The researchers suggest that the mechanism in which LM11A-24 and LM11A-31 prevent oligodendrocyte death when exposed to proNGF is by limiting the binding of proNGF to the $p75^{NTR}$. As oligodendrocytes are reasonable for myelination of the neurons in the central nervous system, a decrease in oligodendrocytes would affect neuronal communication and eventually neuronal death. The $p75^{NTR}$ has many downstream affects that must be accounted for as a target for treating Alzheimer's Disease. Multiple types of molecules can bind to the $p75^{NTR}$ such as: neurotrophins, pro-neurotrophins, Aβ, and other small ligands like the LM11A compounds. Many pathways, for survival or death, are implicated through the $p75^{NTR.}$ The LM11A compounds seem to be able to treat or prevent the $p75^{NTR}$ from activating neuronal death pathways and instead promoting neuronal survival. These compounds can mediate the negative responses from the $p75^{NTR}$.

A limitation of the study evaluating the neuronal protectant effect of the LM11A compounds it that the cells cultures were harvested from the hippocampus region of E16-17 mice (Massa et al 2006). They were stimulated with all compounds/treatments and cell survivability

assayed after only forty-eight hours of plating. The neurons at E16-17 are not as developed as they could be to resemble neurons found in Alzheimer's Disease patients. Those that suffer from AD are older individuals with brains that have had years to develop. No primary neuronal culture is going to be able to survive years in a laboratory setting, but the neurons could have been allowed to grow longer before stimulation occurred. The neurons pictured in their paper do not have neuronal processes like axons and dendrites. Without the neuronal processes it is hard to compare how the underdeveloped neurons react to a condition to the more developed neurons as axons and dendrites are essential structures for the neuronal communication and survivability. Also, if the neurons were grown for a longer amount of time, they could have begun synthesizing endogenous neurotrophins which would not only make the cells less vulnerable to stimulation but also provide a more comparable model to the neurons from a human.

Since LM11A-31 and LM11A-24 have both shown neuroprotectant effects, another matter to consider is if one is a better suited treatment for Alzheimer's Disease. There is a broad range of LM11A compounds the differ structurally, but LM11A-24 and LM11A-31 were compared against each other in series of test in *in vitro* and *in vivo* models (Nguyen et al. 2014). Transgenic mice overexpressing the Aβ precursor protein (Thy1-hAβPPLond/Swe, also known as $A\beta PP^{L/S}$, mice) were treated with LM11A-31 or LM11A-24 at the age at which Aβ plaques are seen in this transgenic model, which is between 3-4 months old. LM11A-31 and LM11A-24 showed a neuroprotectant effect in the AβPPL/S by preventing shrinking of cholinergic neurite length and decreasing of in cholinergic neurite volume in AβPPL/S cells harvested from the basal forebrain. Without the administration of either of the LM11A compounds neurite length was severely shorter in the $A\beta PP^{L/S}$ cell as compared to the wild type cells. This suggests that LM11A compound prevented the neurite dystrophy in the AβPPL/S from developing and

therefore promotes improved neuronal signaling and a potential treatment against Aβ plaque build-up. The researchers saw that the $p75^{NTR}$ was cleaved in cells harvested from the hippocampus of the $A\beta PP^{L/S}$ mice and administered LM11A-31. Based on their results, it appeared that cleavage of $p75^{NTR}$ is also present in wild type mice whether given LM11A-31 or not but $p75^{NTR}$ cleavage is increased when administered LM11A-31 and most increased in $A\beta PP^{L/S}$ mice treated with LM11A-31. This suggests a mechanism of how LM11A-31 could exhibit neuroprotectant effects in Alzheimer's Disease like conditions. The researchers also saw that LM11A-31 effects levels of tau phosphorylation, reactive astrocytes, and microglia in the cells harvested $A\beta PP^{L/S}$ (Nguyen et al. 2014). This indicates that LM11A-31 modulates the $p75^{NTR}$ which has diverse downstream affects the effect neuronal survival. For a disease like Alzheimer's Disease of which there is no set biomarker established as the cause of developing the disease, a drug that targets multiple biomarkers of the disease is an effective strategy in treating it. The researchers did not evaluate how LM11A-24 affected tau phosphorylation, reactive astrocytes, and microglia but they did evaluate how LM11A-24 affected cholinergic neurites. Compared to LM11A-31, LM11A-24 exhibited a smaller neuroprotectant effect in the *in vitro* studies.

While both LM11A-31 and LM11A-24 showed a neuroprotectant effect in preventing neurite dystrophy in cholinergic neurites in the in vitro studies, the in vivo tests suggest LM11A-31 as the more effective LM11A compound (Nguyen et al. 2014). The $A\beta PP^{L/S}$ mice were subjected to four trials of delayed-matching-to place water maze over a series of 6 days. Wild type mice showed similar escape latency times over the multiple days of exposure to the delayed-matching- place test. While $A\beta PP^{L/S}$ treated with either of the LM11A compounds showed lower escape latency times throughout as the days of the maze continued, the mice

treated with LM11A-24 had a smaller decrease in escape latency times than the mice treated with LM11A-31. The AβPP$^{L/S}$ mice treated with LM11A-31 had similar escape latency times compared to the wild type mice. In the fourth trial of the maze, LM11A-31 treated AβPP$^{L/S}$ mice decreased the escape latency time to about half of the AβPP$^{L/S}$ untreated mice. During trial four of the maze, the LM11A-24 treated AβPP$^{L/S}$ mice and the AβPP$^{L/S}$ untreated mice had almost the same escape latency time. This indicates that while LM11A-31 and LM11A-24 may show similar potential as a treatment for Alzheimer's Disease at a cellular level, behaviorally they differ. LM11A-31 appears to be the superior LM11A compound in reference to its ability to prevent neuronal death and deficits in cognitive behavior. While LM11A-24 did not necessarily have zero neuroprotectant effect, LM11A-31 showed to act more as a neuroprotectant and therefore more useful as a drug for targeting p75^{NTR} mediated neuronal death and deficits in cognition.

There are biases within this study as most data was collected from mice only treated with LM11A-31 and not both LM11A compounds. The *in vitro* tests did not show any data regarding tau clusters, levels of microglia and reactive astrocytes in AβPP$^{L/S}$ cells treated with LM11A-24(Nguyen et al. 2014). Also, in the pathways in which the AβPP$^{L/S}$ mice treated with LM11A-24 were not present in the delayed-matching-to place maze trials. The pathways that the AβPP$^{L/S}$ treated with LM11A-31 were drastically different than the AβPP$^{L/S}$ untreated mice. The escaped latency times for all trials/days from AβPP$^{L/S}$ mice treated with either LM11A-31 and LM11A-24 were both presented but seeing the pathways in which the mice travelled could support one LM11A compound over the other as the superior LM11A compound. Inconsistently of within the data analysis of which LM11A compound is administered creates a bias that effects which LMA11 did perform better and suggested to be a more useful drug treatment against Alzheimer's

disease. However, -31 this bias does not remove the neuroprotective ability indicated by LM11A-31 and therefore makes it a potential compound in a multi-compound treatment for AD.

Resveratrol and Sirt1 protein

Resveratrol is an organic phenol compound that was first isolated from the plant *Veratrum grandiflorum* by the Japanese scientist Dr. Michio Takaoka in 1939 (Pezzuto 2019). It is found is many foods including peanuts, grapes, and wine. Resveratrol does have cis and trans configuration, but it is the trans isomer often found in these foods and used in studies. This is because the trans isomer is theorized to be more stable than the cis configuration therefore binds to proteins more effectively. Some have suggested that a diet including foods containing Resveratrol may be beneficial because Resveratrol has shown an array of health benefits from an anti-bacterial to cancer treatments and has anti-inflammatory properties (Salehi et al. 2018). However, the studies in which Resveratrol has indicated any potential health benefits have used Resveratrol at much higher concentrations than found naturally in the foods containing Resveratrol (Pezzuto 2019). Therefore, it is not as simple to directly correlate a Resveratrol enriched diet with any of potential affect Resveratrol may have on certain ailments.

The compound Resveratrol has been studied as a treatment for an array of illnesses including types of cancers, cardiovascular disease, diabetes, and neurodegenerative diseases. Animal studies have indicated a loss in tumor formation of multiple cancers such as a breast, colorectal and pancreatic cancers through an oral administration of Resveratrol (Carter et al. 2014). Though in each study the researchers theorize a different target or pathway in which Resveratrol may have prevented the formation of tumors of the respective cancer. For instance, the authors of the study on breast cancer suggest that Resveratrol binds to estrogen receptors acting as an agonist and antagonist (Bowers et al. 2000). In one experiment on colorectal cancer

rats injected with 1,2-dimethylhydrazine (DMH) to stimulate colon cancer and fed a Resveratrol enriched diet showed a decrease in aberrant crypt foci (ACF), a precursor of colon cancer (Sengottuvelan and Nalini 2006). The pancreatic cancer study yet again suggests a different method in which Resveratrol decreased tumor growth; theorizing that Resveratrol inhibits ERK, Pi3K, AKT, FOXO1 and FOXO3a proteins (Roy et al. 2011). Resveratrol seems to have an affinity for many different proteins and shows a vast array of ways it might be useful as a therapeutic treatment for many different diseases. Its ability to bind to so many proteins has its advantages and disadvantages when studying Resveratrol as a neuroprotectant. The main disadvantage being that it could be difficult to find a pathological way in which Resveratrol acts as a neuroprotectant. For instance, Resveratrol may influence pathways attributed to the aggregation of Aβ plaques and/or the neuronal tangles caused by hyperphosphorylation of tau as well as have effects on pathways independent of Aβ plaques and tau tangles. However, this disadvantage leads to an advantage of a compound with such low specificity. Resveratrol's ability to bind to many different proteins may present a therapeutic treatment for Alzheimer's Disease because it could affect unknown causes of the disease at the moment. It may not just be the Aβ plaques or the hyperphosphorylated tau tangles that lead to the neurotoxicity and eventual development of Alzheimer's Disease. Perhaps a multitude of pathways are involved and a drug that targets all pathways without causing toxicity itself may be a valuable therapeutic technique.

One target that Resveratrol has been linked to is the Sirt1 protein which is thought to play in Aβ metabolism. In a study done to observe the effect tread mill exercise on $A\beta_{1-42}$ and $A\beta_{1-40}$ degradation induced by Sirt1, Resveratrol was not the focus of the experiment, but the researchers provided a potential pathway in which Sirt1 activation leads to a decrease in Aβ accumulation (Koo et al 2017). The researchers saw that mice subjected to treadmill exercise

saw an increase in Sirt1 expression which they theorized lead to a decrease in ROCK-1 and Aβ -cleavage, an increase in Aa cleavage, RARBd and ADAM-10 expression, ultimately causing a decrease in Aβ production. In this study Sirt1 is also mentioned to affect other proteins to encourage a nonamyloidogenic pathway, an increase in PC1a and a decrease in BACE-1 (Koo et al 2017). While this study does not suggest that Resveratrol follows this exact pathway to limit Aβ accumulation, it presents a potential method Resveratrol may show neuroprotective affects. If activating or increasing Sirt1 expression is the main goal, using a compound to do so may be easier for patients of Alzheimer's Disease who are typically elderly and may have difficulty exercising to an amount that can reproduce the effects seen in the treadmill mice. Also, finding a drug that can activate/increase expression of Sirt1 is easier to manage and control.

Resveratrol can act as an ant-inflammatory making it a target for a second hallmark of AD. Streptozotocin is a compound used to model diabetes mellitus and has shown in rats to induce the neuroinflammation leading to neurodegeneration and cognitive deficits (Nazem et al. 2015). In one study a combined model of Alzheimer's Disease and diabetes, utilizing $A\beta_{1\text{-}40}$ and streptozotocin injections, were used to evaluate Resveratrol's abilities to reduce inflammation by measuring the effect on Sirt1 expression which plays a role in inflammatory responses (Ma et al. 2020). The rats were divided into groups and treated orally with Resveratrol and EX527, a Sirt1 inhibitor. The analyze the levels of chemokine IL-1β and IL-6 whose increase is often linked to inflammation in Alzheimer's Disease patients. The researchers report that when their AD/DM rats were treated only with Resveratrol the IL-1β and IL-6 decreased but increased when also stimulated with EX527. The anti-neuroinflammation effects from Resveratrol may have been inhibited by the presence of the Sirt 1 inhibitor. It is unclear if the increase in IL-1β and IL-6 is a response to the presence of Aβ plaques or a factor that contributes to the production of Aβ

plaques which is emphasized in this study because there was a lack of controls in the experimental design. Stressed rats were stimulated with both $A\beta_{1-40}$ and streptozotocin injections therefore it is inconclusive if the inflammatory and cellular toxicity responses were induced by the $A\beta_{1-40}$ or streptozotocin injections.

Resveratrol has shown some promise against neuronal toxicity associated with Aβ and oxidative stress. In one study Resveratrol protected against neuronal toxicity when stressed with $A\beta_{25-35}$ (20μM) (Han et al. 2004). The researchers employed a pre-co-post-treatment strategy with Resveratrol to examine potential pretreatment, cotreatment, and post treatment protection against neuronal loss from $A\beta_{25-35}$ induced toxicity. They observed the most protection against $A\beta_{25-35}$ induced cell toxicity when cells were treated before being stressed with $A\beta_{25-35}$ although it is unclear why the cells were able to survive more when pretreated with Resveratrol because the cells were only pretreated for 2 hours prior to stimulation and it is unlikely that transcriptions factors were produced that could protect against $A\beta_{25-35}$ induced toxicity. The cells co-treated with $A\beta_{25-35}$ and post-treated with Resveratrol after being stimulated with $A\beta_{25-35}$ for 2 hours did have a higher viability than cells only stressed with $A\beta_{25-35}$, but not as dramatically when the cells were pre-treated. This study indicates that Resveratrol may be useful in slowly the progression from one anatomical region already affected by Alzheimer's disease to another by preventing neuronal atrophy.

Another case in which Resveratrol may show neuroprotective effects evaluated Resveratrol's ability to induced mitophagy to recycle dysfunctional mitochondria produce oxidative stress (Wang et al. 2018). The authors induced cell toxicity through dysfunctional mitochondria using $A\beta_{1-42}$ in PC12 cell culture. Resveratrol treated PC12 cells were seen to have higher cell viability than just stressed $A\beta_{1-42}$ cells and a decrease in mitochondria. The researchers

suggest that the Resveratrol can promote mitophagy to removed dysfunctional mitochondria induced by $A\beta_{1-42}$ toxicity limiting oxidative stress to the cells. It is inconclusive how Resveratrol inducing mitophagy will affect neuronal cultures due to the use of PC12 cells and not neurons directly and if Resveratrol can differentiate between dysfunctional and healthy mitochondria. If Resveratrol can induce mitophagy in dysfunctional mitochondria only in neuronal culture this may be a neuroprotective strategy against $A\beta_{1-42}$ induced oxidative stress. Resveratrol's seems to mainly affect Aβ, Sirt 1, inflammation proteins, and oxidative stress. Resveratrol being able to potentially target 3 hallmarks of AD and promote neuroprotective properties suggest it may also play a crucial role in a combined treatment strategy.

Methylene Blue

Methylene blue (MB) is compound used as a dark blue dye as well as for medical purposes. MB has been used to treat multiple ailments such as cancer, Parkinson's Disease and Alzheimer's Disease and thought to play a role in transferring an extra electron in the mitochondrial electron transfer chain increasing metabolism and affect biomarkers of Alzheimer's Disease such as a tau aggregation (Yang et al 2017). One way MB has been suggested to exhibit a neuroprotective effect and therefore a potential treatment for neuronal death induced by AD hallmark, hyperphosphorylation tau, is by decreasing NFT (Hochgräfe et al. 2015). MB was reported to decrease Tau aggregation in pro-aggregate human full-length Tau transgenic mice through oral administration and decrease cognitive decline via decreased pathway length during a Morris water maze test. However, MB's positive effect was not able to completely reverse any cognitive damage done by the NFT and tau aggregation present in the Tau transgenic mice and MB's neuroprotectant effect is only present for a short period of time before the cognitive decline increased once again. This indicates that MB may be able to limit

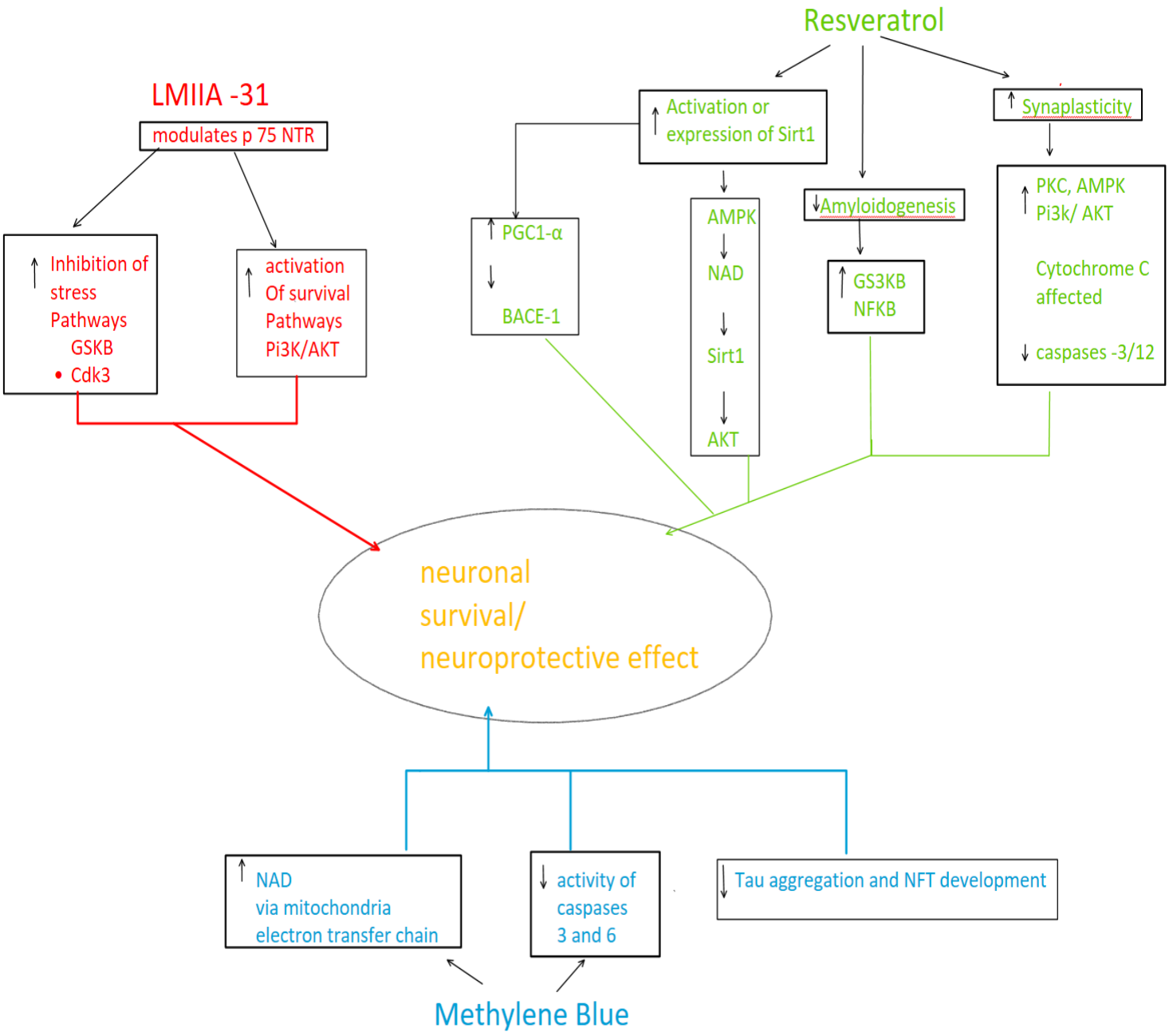
tau phosphorylation and aggregation preventing NFTs from developing for a period of time, but only until a certain point and would require early detection of AD to be helpful for patients. As patients can develop AD biomarkers long before symptoms become present, MB by itself may not provide the strongest neuroprotectant effect. However, MB could be used in conjunction with another neuroprotectant compound that is able to modulate later stages of AD biomarkers while MB inhibits NFT development.

Another method that MB may be involved in to elicit a neuronal protective affect is by limiting stress induced by caspase pathways. Methylene Blue was shown to inhibit the activity of caspase 6 overexpressing mice and thought to reverse cognitive and synaptic decline typically induced via caspase 6 expression (Zhou et al 2019). Transgenic mice with a knock-in of the Cre gene to express human caspase 6 were subjected at 18 months old were given a daily orally administered dosage of Methylene Blue for one month and then subjected to behavioral exams (novel object recognition, Barnes maze with probe test, and open field test) and their caspases 6 expression levels were measured. There were two different types of transgenic mice that either removed a stop codon between human Casp6 and / a promoter to express human Casp6 (type I ACL KI/Cre) or included the stop codon (type II ACL/G Kre/Cre) and wild type groups with and without KI or Cre (Zhou et al 2019). The researchers noted that KI/WT and ACL/G KI/Cre behaved the same on the Barnes maze, NOR and OFT so their data is grouped and reported together and referred as ACL/G. Overall mice overexpressing Casp6 and treated with MB reported an improvement in cognitive function as the researchers suggest a decrease in primary latency time, error, and an increase in total number of visits to the target hole in the Barnes maze and increase discrimination index in the NOR results. The model system does raise some questions as there was no difference in behavior between ACL/G KI/WT and ACL/G KI/Cre

mice indicating the Cre insertion had no effect on the ACL/G mice but as the ACL mice also exhibited similar results the importance of these results is less concerned with the genotype but with MB neuroprotective properties. Further studies using a non-transgenic model system could provide more insight as to if MB does have the ability to reverse cognitive decline as claimed by this study. Though, it is more likely that MB could prevent further cognitive decline rather than reverse damage induced by Casp6 and if prescribed to patients in the early stages of Alzheimer's Disease then could stop the progression of the disease.

Besides behavioral tests, the researchers also analyzed the inhibition of Casp6 in MB treated mice using two-photon microscopy (Zhou et al 2019). The ACL/G mice and WT/WT were given vehicle, MB, or Casp6 inhibitor z-VEID-FMK. Casp6 fluorescence levels of MB and z-VEID-FMK in both WT and ACL/G mice were incredibly low indicating inhibited Casp6 activity compared to vehicle ACL/G mice. This suggests that after mice who had been over expressing Casp6 for 18 months with only one month of MB treatment can show similar Casp6 to wild type mice not expressing Casp6. The researchers theorize that inhibiting activity of Casp6 but not a decrease in Casp6 expression contributed to the behavioral improvements seen in the ACL/G mice as there was not a difference in expression levels between vehicle and MB treated (Zhou et al.2019). Distinguishing that MB may affect protein activity instead of expression levels is important to understanding how its individual neuroprotective effect are produced but also how it may contribute to a synergistic pathway between LM11A-31 and Resveratrol.

Potential Pathways affected by LM11A-31, Resveratrol, and Methylene Blue



Schematic 2: Potential pathways LM11A-31, Resveratrol, and Methylene Blue could follow to promote neuronal survival. This schematic is meant to illustrate the different pathways each drug could modulate and how they may affect similar pathways that could serve as targets to treat AD.

Research Proposal

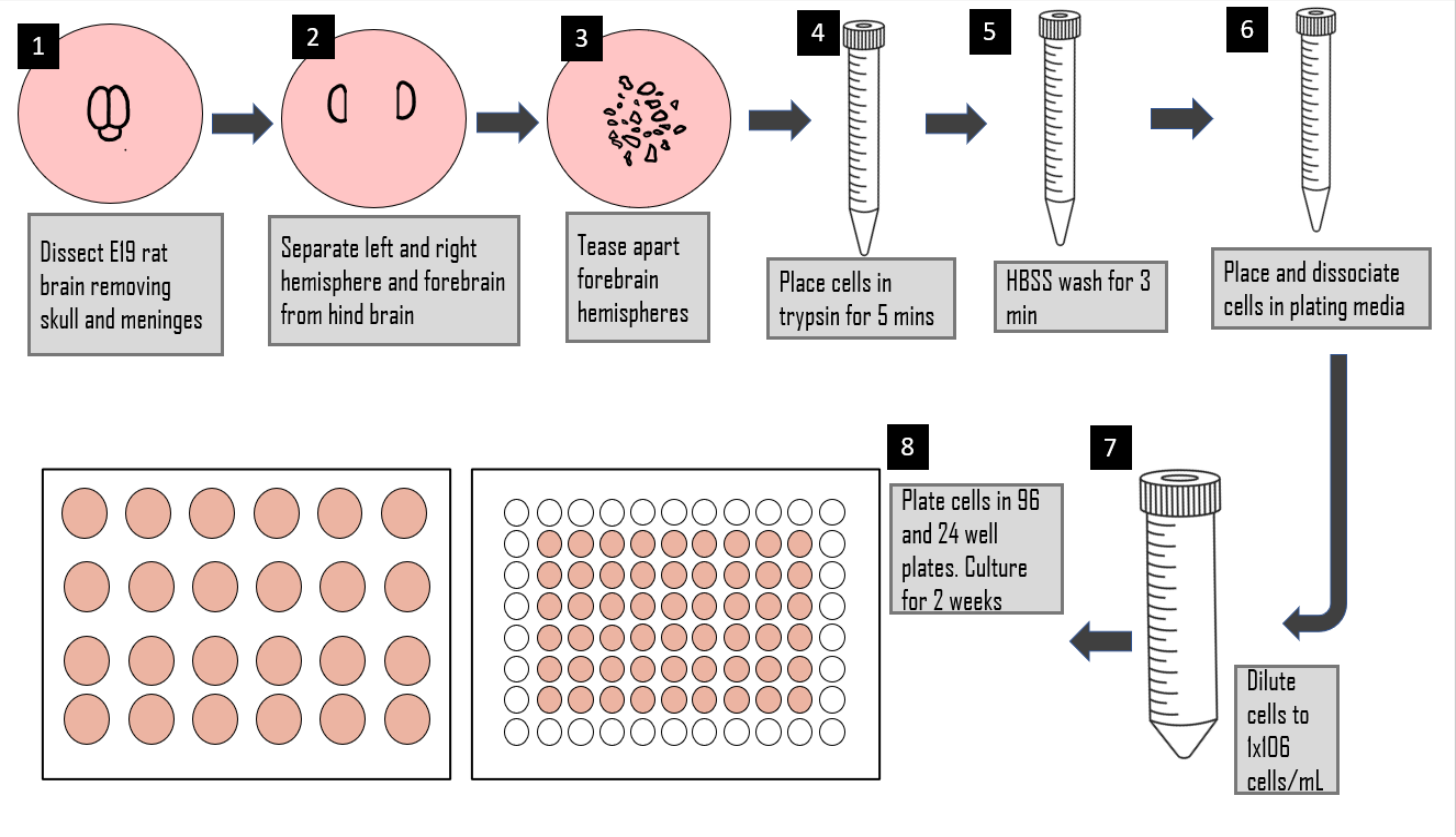
In my study I will evaluate three compound's neuroprotective properties and their overall ability to treat AD hallmarks, Aβ accumulation, hyperphosphorylated tau, inflammation, and oxidative stress. The compounds in question, LM11A-31, Resveratrol, and Methylene Blue have individual some evidence supporting their effects on protein pathways exhibit neuroprotective effects; however, similarly to current drug treatments available to AD patients their neuroprotective effects were limited and inconclusive. It is proposed that a combined treatment strategy using all three therapeutics will produce a greater neuroprotectant effect together against stressors such as FAB and NMDA, than each one individually.

All studies were be done using an in vitro cell culture model system in which cell survival and microtubule stability was measured. Due to the COVID-19 virus halting in person research for a period of time, no protein analysis was able to be completed. A majority of the primary literature mentioned previously use an in vivo model and mainly administer the compounds orally. However, an in vivo model limit the ability to see how the drugs are affecting the hallmarks of AD at a molecular level. All of the compounds, LM11A-31, Resveratrol, and Methylene Blue have not shown conclusive data suggesting they are an effective treatment for AD patients individually. To determine if they are able to protect against neurodegeneration induced by stressors associated with AD, it would be helpful to see at the molecular level their potential neuroprotective effect. There is not an existing study that evaluates the combined treatment of specifically LM11A-31, Resveratrol, and Methylene Blue, I present a pilot study examining their potential neuroprotective effect against stressed neuronal cultures.

Methods:

Dissection and cell culturing

Forebrain neurons were harvested from day 19 embryonic rat brains. The E19 brains were removed from the skull and the meninges were excised off the tissue. If meninges were not removable from sections of tissue this tissue was discarded to prevent cell death caused by cultures exposed to meninges. Forebrain hemispheres were separated, and the hindbrain discarded. Tissue was teased into pieces and placed in trypsin and water bath (37℃) for 5 minutes. The tissue was placed in an HBSS wash for 3 minutes twice before being transferred to plating media. Cells were dissociated and counted using a hemocytometer to calculate volume needed to dilute cells to $1x10^6$ cells/ml. Cells were diluted using plating media and plated in 24 well and 96 well plates. Plating media was removed after an hour from original plating and replaced with GM to prevent neuronal death induced by FBS in the plating media. Approximately half of the GM was replaced every other day for 2 weeks until cells were ready for stimulation.

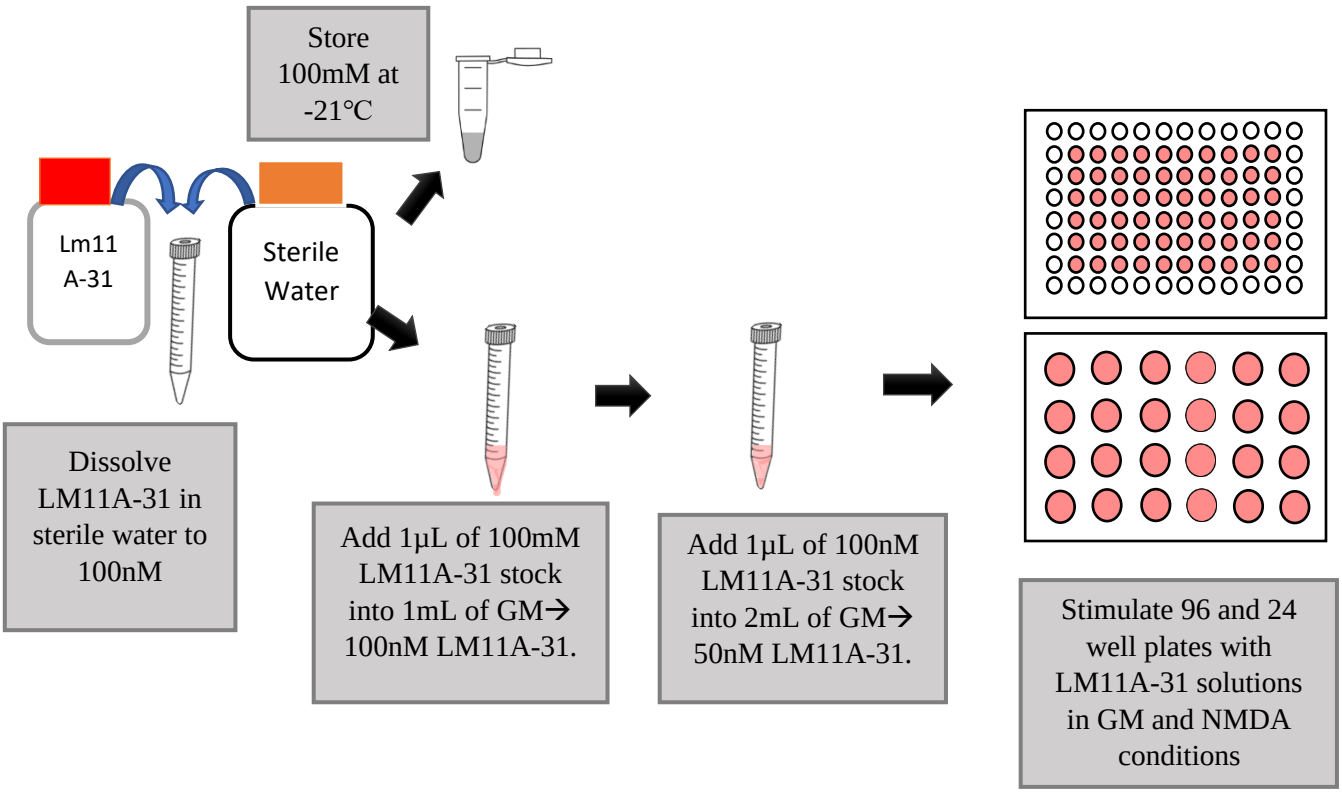


Schematic 3: Illustration of dissection and growing neuronal culture procedure. This schematic highlight the procedure in which the neuronal cultures are obtained and kept alive till maturity until ready for drug stimulations.

LM11A-31, Resveratrol, Methylene Blue, and combination solutions

Concentrations were based on previous studies and a concentration curve for each compound. The following concentrations were chosen based on which concentration in the curve indicated the most neuronal survival when cells were treated with the compound and stressor, 200µM NMDA: LM11A-31 (50nM), Resveratrol (20µM), Methylene Blue (100nM). The cells were stressed with 200µM NMDA because lower concentrations (100µM) induced high survival in cells treated with just stressor indicating higher concentration was needed. One test, LM11A-31 concentration curve, was done using FeSO4(7.95mg), amyloid beta (1µM Aβ) L-buthionine(133.5mg) (FAB) diluted into 50mL of growth media (GM) as a stressor but NMDA

was used as the stressor for following experiments. The FAB was diluted further into 1:4 ratio with GM to make it a working solution for cell culture. Each compound was dissolved in sterile water (LM11A-31 and Methylene Blue) or DMSO (NMDA and Resveratrol) and sterilized through a syringe filter. Stock solutions were kept at -21 to use as needed. Serial dilutions were required to dilute the compounds down to the small concentrations. LM11A-31 solutions were made before each stimulation because the diluted and refrigerated LM11A-31 solutions became unstable after a few days from original dilution. Dilutions for each compound was made using GM or 200μM NMDA. Cells treated with a solution containing NMDA were replaced with GM diluted compounds after 30 minutes. Each drug was tested individually for the neuroprotective ability and then combined treatments were analyzed. GM only and NMDA only treated cells served as positive and negative controls, respectively. Combined treatments include combined treatment 1(LM11A-31 and Methylene Blue), and combined treatment 2 (LM11A-31, Methylene Blue, Resveratrol. The cells were stimulated for 1-2 days before MTS assay and ICC analysis was conducted.



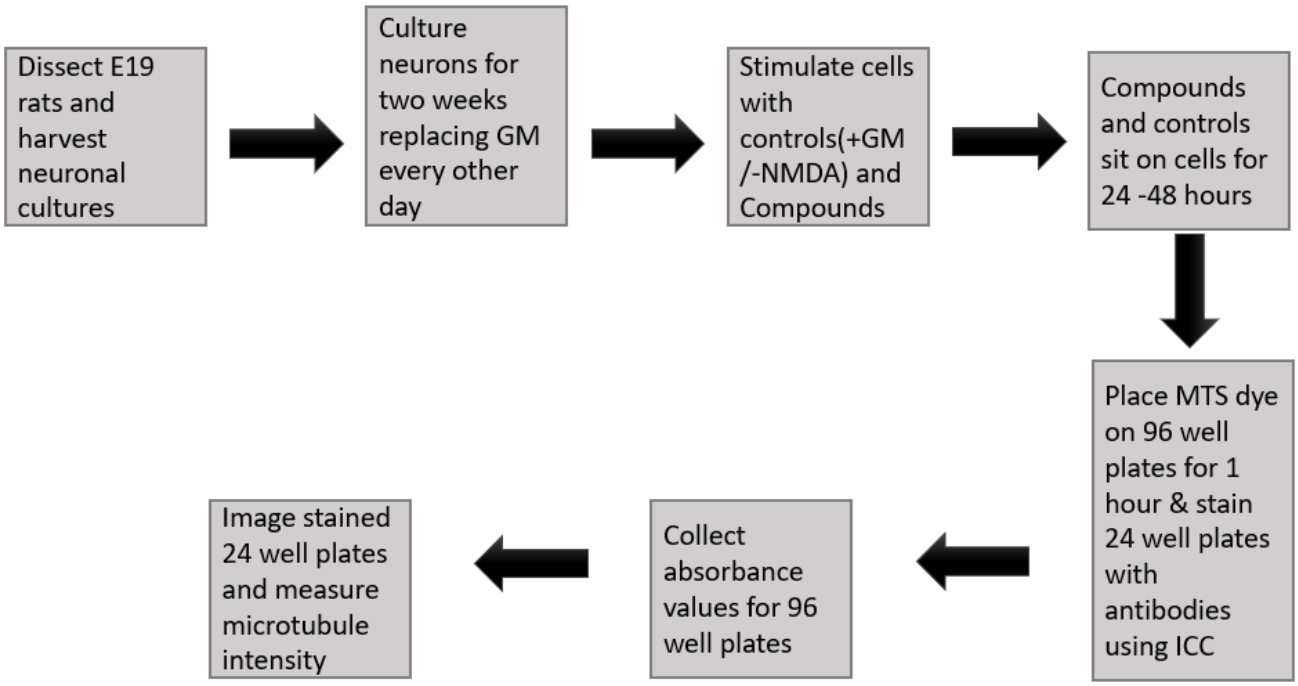
Schematic 4: Illustrates how one of the compounds, LM11A-31, was prepared before stimulating the cells with said compound. Preparation of resveratrol and methylene blue followed similar procedures.

MTS Assay and ICC

One to two days after 96 well plates were stimulated, the compound, GM, and NMDA solutions were removed and replaced with MTS. The MTS was placed on the cells for at least an hour but no longer than four hours. Immediately after the MTS had been on the cells for an appropriate amount of time, the absorbance of the wells were measured using either a plate reader or Amersham Imager and associated absorbance measuring programs. The absorbance of all wells treated with the same conditioned were averaged and normalized to the wells treated with only GM.

The 24 well plates were stained using Immunocytochemistry (ICC) techniques. 4% paraformaldehyde was placed on the cells for 20 minutes, collected discarded appropriately and washed with PBS solutions 3 times. Each PBS solution was placed on the wells for 3 minutes at a time. 0.5 % Triton-100 was placed on the wells for 10 minutes followed by 1 PBS wash. The plates were incubated with primary antibody anti-acetylated tubulin on a shaker covered with aluminum foil for at minimum 1 hour. The primary antibody was recollected and plated were washed briefly with PBS 3 times. Wells were divided into groups incubated with FITC or Cy3 for at minimum 1 hour. If plates showed little but some visible fluorescence under the Nikon Eclipse fluorescent microscope, the plates were incubated again with primary and secondary antibodies. Images of each 24 well plate were taken at different points of the well at random to prevent biased results from what was expected or predicted. Microtubule structural intensity was measured by calculating the relative intensity for whole and binary image and the area of fraction percentage.

Timeline for procedure

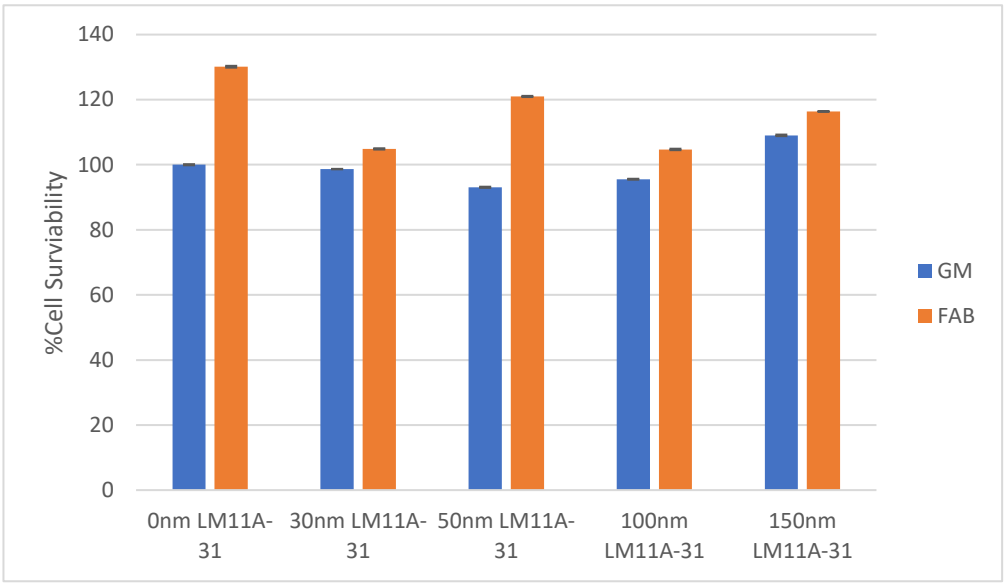


Schematic 5: Timeline of procedure of all experiments. All following experiments were subjected to this procedure for the duration of this study with minor adjustments in protocol.

Results:

Multiple different tests were done in order to evaluate how LM11A-31, Resveratrol, and Methylene Blue affected neuronal cultures when exposed to GM, and either FAB or NMDA conditions. Types of tests done included concentrations curves for LM11A-31 and Resveratrol (figures 1 and 4), a concentration comparison for NMDA (figure 2), and two combined treatments: combined treatment 1 (LM11A-31/Methylene Blue, figure 3) and combined treatment 2 (LM11A-31, Resveratrol, and Methylene Blue, figure 5). MTS assays and immunocytochemistry (ICC) was used to measure cell survivability and microtubule stability, respectively. NMDA and FAB demonstrated little to no stressors effect illustrated by high levels of cell survivability and microtubule stability levels throughout most of this study. As such there was no neuroprotectant effect from the compounds LM11A-31, Resveratrol, and Methylene Blue. Resveratrol seemed to produce an adverse effect as demonstrated by the decrease in cell survivability and microtubule stability when cells were exposed to NDMA and Resveratrol only (figure 4). Due to a small number of trials completed for each experiment, inferential statistics were not able to be completed and therefore not statistically significant or insignificant results are listed. Instead, statistical analysis was done using descriptive statistics. For all experiments containing 2-3 trails, descriptive statistics were obtained from normalized values of cell survivability, area of fraction, and microtubule intensity. If an experiment had only 1 trial, descriptive statics was not applicable.

A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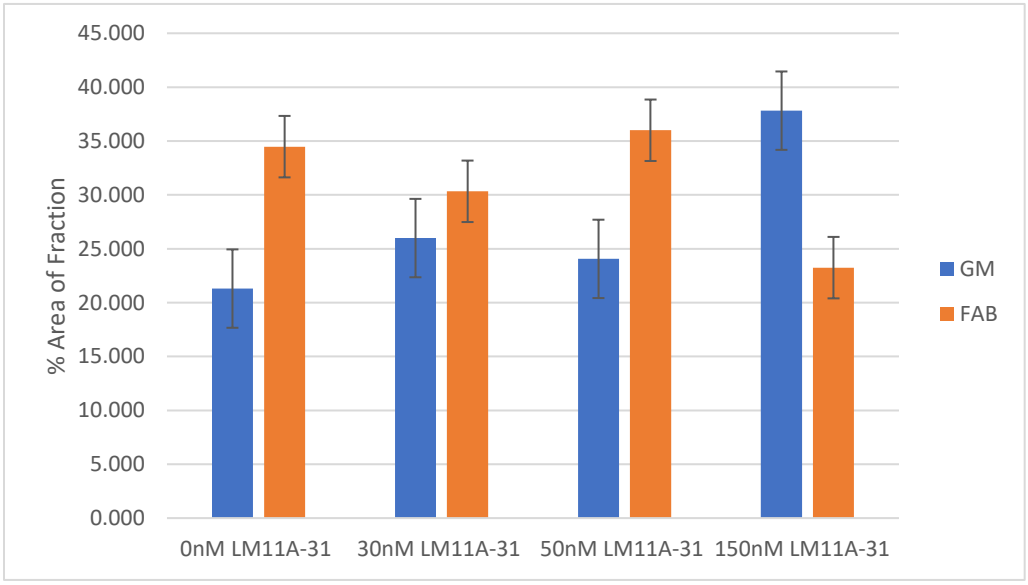
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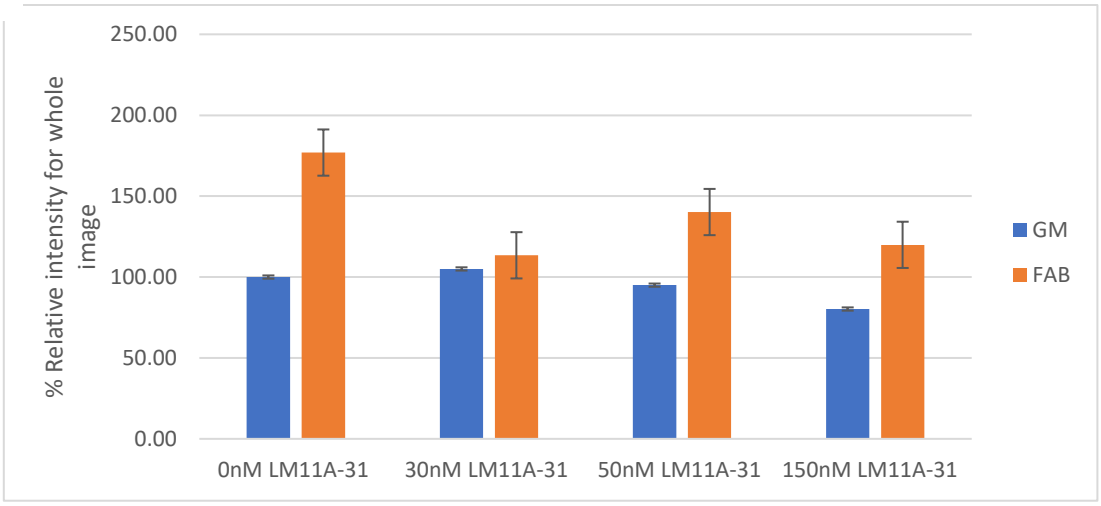

Figure 1: Concentration curve of LM11A-31 using MTS assay and ICC when exposed to FAB. A. MTS assay results measuring % cell survival rates at various LM11A-31 concentrations. N (total number of trials) =1. ICC was used to measure microtubule stability by analyzing (**B)** percent area of fraction and (**C)** relative intensity for whole image.

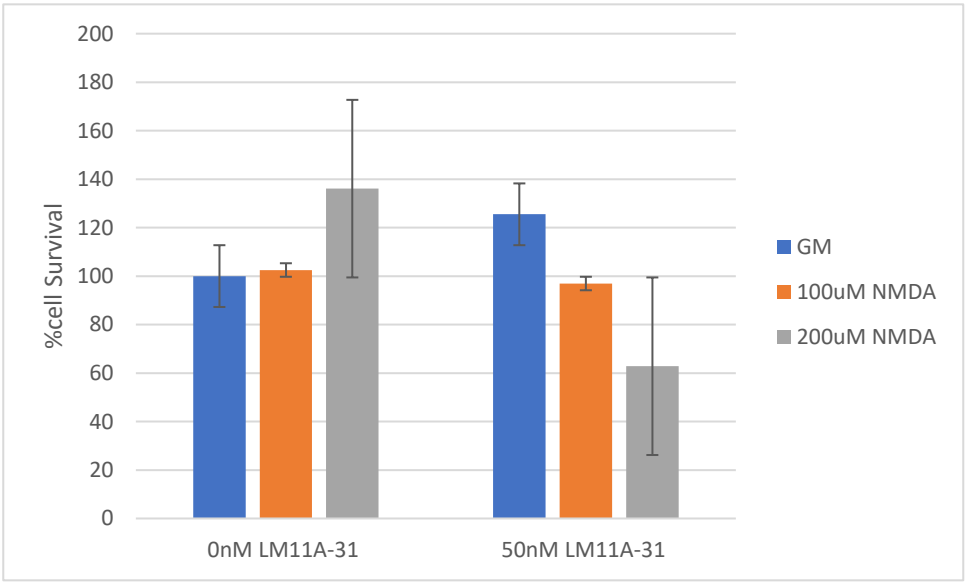

Figure 2: Cell survivability when treated with LM11A-31 and 100uM or 200uM NMD. N=1. MTS assay measuring absorbance of MTS dye. This indicated the cell survivability rates when cells are treated with 50nM LM11A-31 and exposed to 100uM NMDA or 200uM NMDA.

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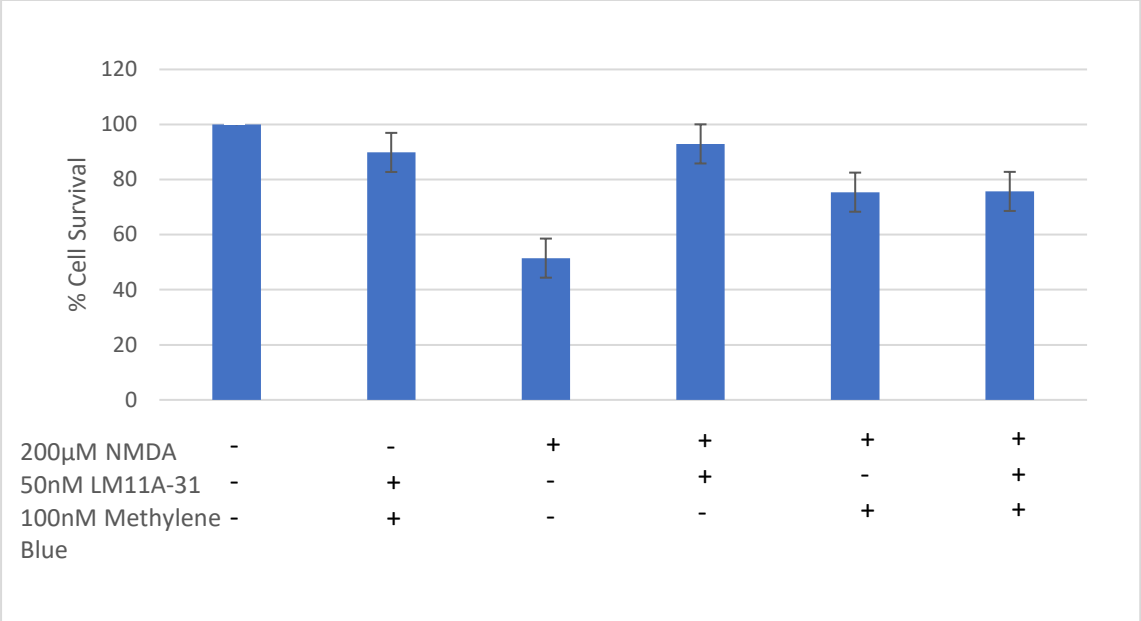
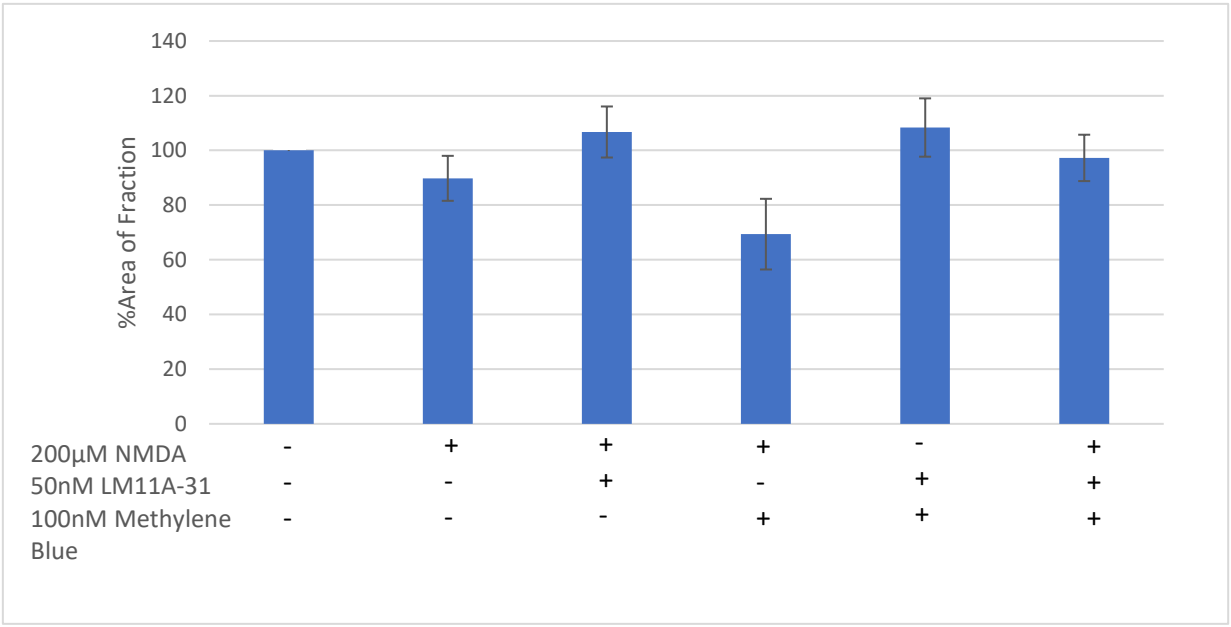


Table 1: Descriptive Statistics of cell survivability trials where cells were exposed to NMDA, LM11A-31 and Methylene Blue

Condition	Average	Standard Deviation	Median	Standard Error
200µM NMDA – LM11A-31- Methylene Blue -	100	0	100	0
200µM NMDA – LM11A-31+ Methylene Blue +	89.86873	25.54991616	89.86873	18.06651897
200µM NMDA + LM11A-31- Methylene Blue -	51.47984	58.31055942	51.47984	41.23179198
200µM NMDA + LM11A-31+ Methylene Blue -	92.96078	2.146368829	92.96078	1.517711954
200µM NMDA + LM11A-31- Methylene Blue +	75.43439	21.67875056	75.43439	15.32919153
200µM NMDA + LM11A-31+ Methylene Blue +	75.70913	40.41234396	75.70913	28.57584246

B



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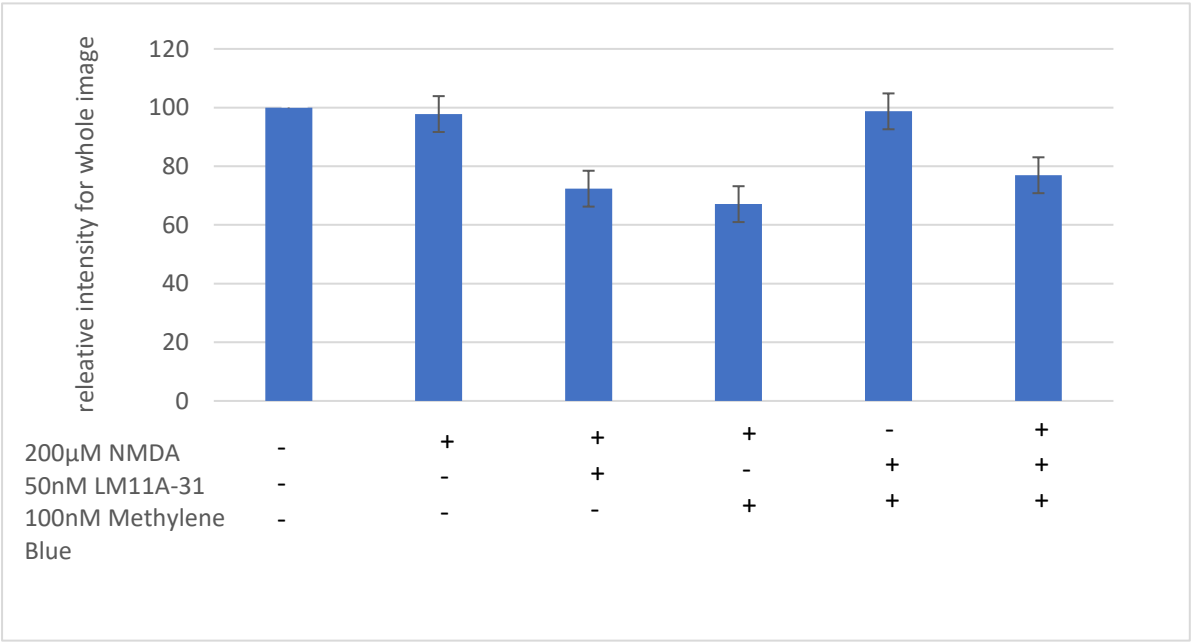
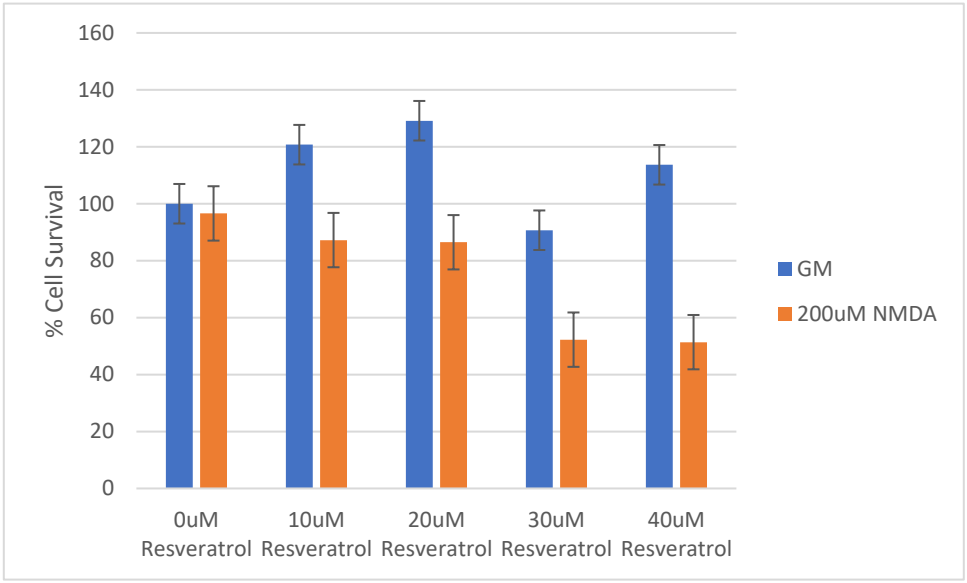


Figure 3: Cell survivability and microtubule intensity when cells are exposed to combined treatment LM11A-31/methylene blue and 200uM NMDA. A. MTS assay results measuring % cell survival rates of cells treated with NMDA, LM11A-31 and/or Methylene Blue. N (total number of trials) =2. Table 1: Descriptive statistic of normalized value from the three trials averaged in figure 3A. ICC was used to measure microtubule stability by analyzing (**B)** percent area of fraction, (**C)** relative intensity for whole image. N=1

A


160
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20uM Resveratrol
30uM Resveratrol
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200uM NMDA

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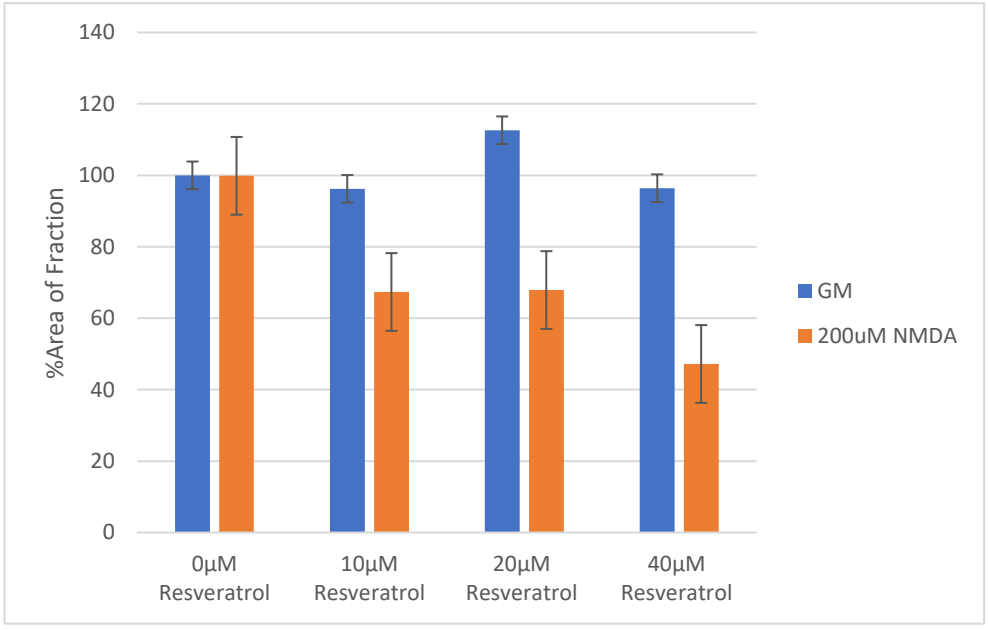
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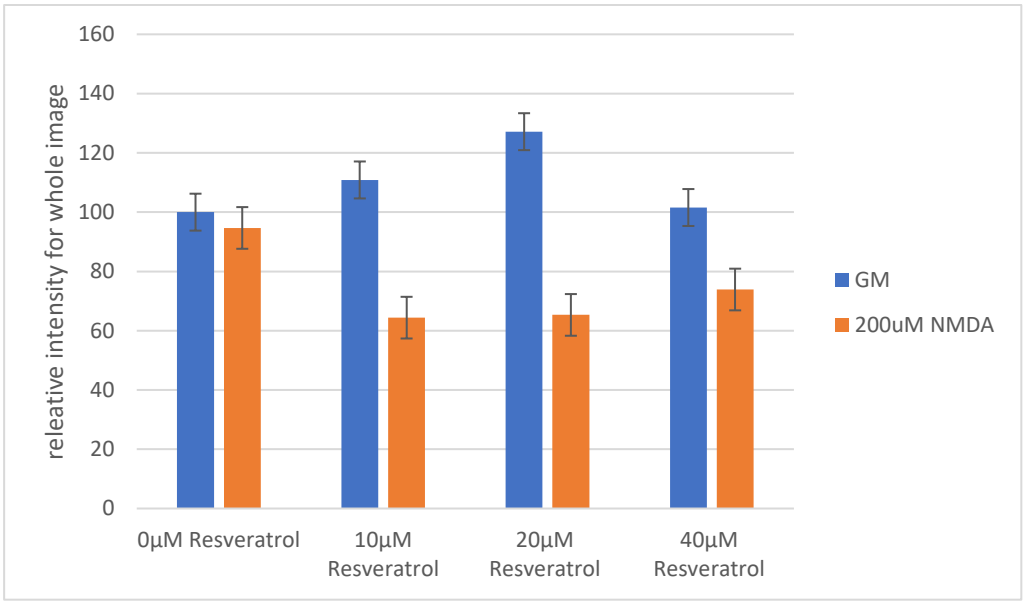

Figure 4: Resveratrol concentration curve when exposed to 200uM NMDA. **A**. MTS assay results measuring % cell survival rates at various Resveratrol concentrations. N (total number of trials) =1. ICC was used to measure microtubule stability by analyzing (**B)** percent area of fraction, (**C)** relative intensity for whole image. N=1

A

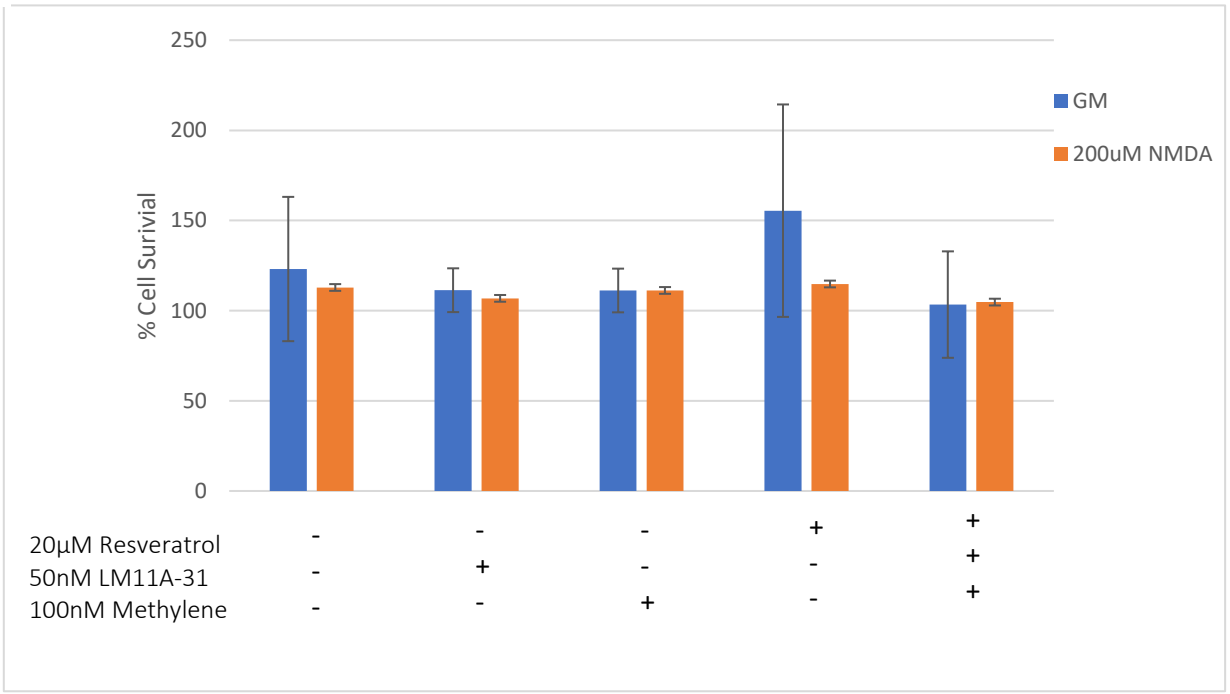

Table 2: Descriptive Statistics of cell survivability trials where cells were exposed to NMDA, Resveratrol, LM11A-31 and Methylene Blue				
Condition	Average	Median	STDEV	Standard error
0 Res/0 LM/ 0 MB	100	100	0	0
50nM LM	99.60252665	97.76398557	14.7074	8.491323763
100nM MB	101.9354803	99.41494629	7.656157	4.420284504
20µM Res	115.5371767	116.932545	11.39171	6.577006112
NMDA	94.22470691	89.82263191	8.70655	5.02672918
LM/NMDA	91.50006078	94.79653309	7.764121	4.482617268
MB/NMDA	88.61175255	85.49919789	11.29059	6.518627825
Res/NMDA	79.22870091	83.14095407	7.914815	4.56962059
Res/LM/MB/NMDA	75.54563716	73.70359482	13.0556	7.537656963
Res/LM/MB/GM	78.32059033	75.74163798	7.630361	4.405391168

Figure 5. Cell survivability when cells are treated with combined treatment of Resveratrol, LM11A-31 and Methylene Blue when exposed to 200uM NDMA. N (number of trials) = 3. **A**. MTS assay results measuring % cell survival rates of cells treated with NMDA, LM11A-31, resveratrol, and/or methylene blue. **Table 2:** Descriptive statistic of normalized value from the three trials averaged in figure 5.

Discussion:

The goal of my research was to conduct a pilot study evaluating a combined treatment of three compounds (Resveratrol, LM11A-31, and Methylene Blue) ability to combat stress on cultured neurons. I harvested neuronal cultures from embryonic (E19) rats and cultured the cells for two weeks before running stimulations. I used an invitro model system is because I wanted to access how these compounds performed as a treatment at a molecular level. Previous research using LM11A-31, Resveratrol and Methylene Blue has been done primarily using in vivo models, which does explore questions that are not answerable when using an in vitro model. An

in vivo model has the advantage of observing behavioral effects induced by the drugs and potentially how the drugs may affect the behavior of a human AD patient. However, in order to treat the behavioral and cognitive symptoms of AD you have to target the cellular and molecular hallmarks of AD that eventually lead to the behavioral and cognitive symptoms. Patients can begin to develop these hallmarks (i.e., Aβ accumulation and hyperphosphorylated tau) years before the behavioral and cognitive impairment is evident. This is where an invitro model presents an advantage over the in vivo models. Perhaps there has yet to be an effective drug treatment for AD because we do not understand how molecularly the drugs can affect the neuronal networks. Especially for this pilot study in which to my knowledge none of these drugs have used together in the method I present; an in vitro model is more beneficial than an in vivo model.

To perform my experiments, I used an in vitro model system by culturing neurons harvested from E19 non-transgenic rats. I harvested neurons from a non-transgenic rat because transgenic rats would induce AD like conditions in the model system that would be comparable to only the familiar form of AD and not the sporadic form. As the sporadic form is the more common type of AD and due to the high failure rate for drugs currently on the market that have primarily tested in transgenic models, a non-transgenic model may show a more accurate representation of the neuroprotective ability of LM11A-31, Resveratrol, and Methylene Blue. The neurons were cultured from E19 rats because if they were cultured earlier their brains would be too small to dissect and any day later the rats would have been born. While the rats would have been bigger and potentially easier to dissect, the number of neurons each rat contained would have decreased compared to the E19 rats. If the rats had been born, they would have had an increase in glial cells and potentially lower concentrations of neurons making it difficult to

count cell survivability using the MTS assay. Glial cells help increase cell survivability in the brain naturally and exist in cultures grown from the E19 rats which are counted in the MTS assay therefore affecting the results. However, the glial cell number is lower in neuronal harvested from the E19 rats, and the ICC test was used to combat glial cell bias. The antibodies used in the ICC, FITC and CY3, only stained of the microtubules of neurons and not the glial cells therefore only measuring microtubule stability and cell survivability of the neurons. The drugs used in this study were thought to only affect neuronal survivability and not glial cells.

Although for a pilot study an in vitro model presents advantages over an in vivo model, this does not indicate that my model system was without flaws. The major flaw I suspect implicated my results is the compound that was meant to serve as a stressor throughout my stimulations did not stress the cells. Ideally the goal was to see if the drugs, especially the combination of all three drugs, would be able to combat stress induced by a compound that typically exhibits strong levels of neuronal death when placed on the cells. However, the two compounds I used to try to stress the cells did not do so. This could be due to a couple of reasons. The first compound I used that was supposed to act as a stressor and kill the neurons, was FAB which contains the amyloid peptide and is thought to be a way to induced AD in non-transgenic rats (Lecanu and Papadopoulos 2013). I used this only when stimulating the cells with various concentrations of LM11A-31 (Figure 1). At minimum, the cells exposed to only FAB should have shown low cell viability and microtubule stability, but this was not the case as demonstrated by my results. The cells treated with FAB had higher cell survivability and microtubule stability than the GM only condition. This suggests that the FAB did not act as a stressor but kept cells more alive than GM. This result was also seen when using the NMDA as the stressor compound as demonstrated in figure 2. Some reasons why the stressor compounds

did not stress the cells are because the cells could have been mostly dead, completely dead and only glial cells remaining, and/or plated at lower concentrations than they were meant to be. If cells were plated at low concentrations, they could die off fairly easily throughout the two-week growing period and normalizing the raw data per condition may show FAB or NMDA cells at a very high cell survivability rate but in reality, all cells in the plate are nearly dead and just a bit more alive than the GM conditions skewing the data. Also, the amyloid peptide and NMDA are found in the brain naturally suggesting positive reasons to produce these compounds. It is the excessive use of these compounds that is toxic to cells. If the cells were mainly dead at time of stimulation, the FAB and NMDA may have presented stimulation necessary to boosted what little cell survivability was left.

Another reason why the stressor compounds did not stress the cells is due to the concentrations within the compounds. Other students in the laboratory reported difficulty stressing their cells using FAB and since my goal was to evaluate if LM11A-31, Methylene Blue, and Resveratrol neuroprotective abilities in a preliminary study, I chose to use a single compound stressor (NMDA) to eliminate testing various concentrations of the multiple compounds found in FAB. I tested NMDA at two concentrations (100µM and 200µM) with LM11A-31 to see if the cells would be stressed more with a higher or lower dose of NMDA as well as if LM11A-31 could combat the different levels of neurotoxicity (figure 2). As the cells exposed to 200µM NMDA and no LM11A-31 had the highest cell survivability, I expect the issue with getting the NMDA to stress the cells mentioned previously were present in these trials as well. I continued to use 200µM NMDA in the following tests to provide more trials in evaluating 200µM neurotoxicity. In other tests, cells exposed to 200µM only did show a decrease in cell survivability, though not a huge decrease. The NMDA was only allowed to

stimulate the cells for an hour during each test including any conditions that contained NMDA and a drug treatment. NMDA previously has shown to completely kill cells to the point at which drugs cannot rescue the cells from the neurotoxicity if left on the cells for longer than a few hours. However, perhaps if the NMDA was left on the cells for a period of time between 1-4 hours there may have been a change in cell survivability.

Before I could test the combination of drugs, I had to determine concentrations per drug using a concentration curve. Finding a concentration of each compound that was appropriate to use on neuronal cultures was difficult due to many resources using an in vivo model. Most of these sources state concentration in mg/kg/day indicating the researcher orally administered the compounds in a specific amount of mg in accordance with the weight of the rat per day. As these concentrations would be too high for neuronal cultures, I had to find smaller concentrations that would not shock the cells. Based on some primary literature using in vitro models LM11A-31 was used in concentrations of 5nM-500nM (Yang et al. 2008). I tested concentrations that fit that range (30nM-150nM) and while the results were inconclusive as the stressor compounds did not stress the cells, I decided to continue the study using 50nM LM11A-31 due to the primary literature suggesting higher concentrations of LM11A-31 may present more of an effect and my own results do not indicate increased cellular survivability and microtubule stability past 50nM (figure 1). The concentration of Methylene Blue was chosen based on previous studies of other students in the laboratory. A concentration curve for Methylene Blue would have been ideal, however due to limit time in the laboratory caused by contamination and COVID-19 restrictions prevented such testing. Researchers suggested that Resveratrol at concentration 25μm protected against oxidative stress in an in vitro model and therefore a range below and above this concentration was chosen to be tested (Bastianetto et al. 2015). A slight increase in cell

survivability and % area of fraction was observed when the cells were treated with 20µM and 200µM NMDA (figure 4). However, this was a slight increase in cell survivability and area of fraction and the cells exposed only to NMDA had a high cell survivability as well. I chose to continue with the 20µM Resveratrol for the remainder of the study to align with primary literature.

Two combinations of the drugs were tested with intention to experiment using more combinations but was limited by time. The first combination of drugs I chose to test was 50nM LM11A-31 and 100nM Methylene Blue. This was one experiment in which the 200µM NMDA did appear to stress the cells has cells only treated with 200µM had a low cell survivability (Figure 3). The cells exposed to LM11A-31, Methylene Blue and NMDA show greater cell survivability and relative microtubule intensity than when cells were exposed to the compounds individually and the drugs independently exposed alongside NMDA. However, it is a small increase using combination 1of the drugs and only indicates potentially a small neuroprotective effect. This might suggest LM11A-31 and Methylene Blue slightly boost each other's potential neuroprotective effect but a third compound, like Resveratrol may boost that neuroprotection further enough to treat the neurotoxicity from the NMDA. However, this is a bold assumption as previously there was difficulty with NMDA acting as a stressor in the concentration curves. Also, this data is from a small number of trials, only two trials. Further tests are needed to tell if LM11A-31 and Methylene Blue work more effectively as a neuroprotective together and if adding another compound could further increase the protection against neurotoxicity.

The second combination of drugs I exposed the cells to be all three drugs (Resveratrol, LM11A-31, and Methylene Blue) and NMDA (figure 5). Before exposing the cells to this combination treatment, the Resveratrol concentration curve suggested that treating cells with

Resveratrol and NMDA produces lower cell survivability and microtubule stability than cells treated with only NMDA (figure 4). Resveratrol having a neurotoxic reaction with NMDA is not support by primary literature. I retested each drug independently with NMDA to see if Resveratrol or any of the compounds have adverse effects when combined with NMDA. There was little to no difference between NMDA only, LM11A-31+/NMDA+, Methylene Blue+/NMDA+, Resveratrol+/NMDA+, and all three compounds+/NMDA+ cell survivability. This makes it inconclusive whether Resveratrol or the combined treatments of the drugs produced either adverse or beneficial effects when exposed to NMDA. Primary literature suggest the drugs have neuroprotective effects when exposed to neurotoxicity but any interactions between these three compounds is not mentioned. The drugs independently have been shown to modulate AD hallmarks and pathways, but little is known about how the drugs react when combined together (schematics 1 and 2). It is also difficult to determine any effects displayed by the drugs using only cell survivability data as this includes both neurons and glial cell and does not provide an explanation as to what is happening the cells themselves. Measuring microtubule stability using ICC was intended but due to contamination of plates and limited time only MTS data was able to be collected.

As the data obtained does not indicate strong conclusive results, future experiments are needed to explore individual neuroprotective abilities of LM11A-31, Methylene Blue, Resveratrol, and combined treatments. Some simpler future directions would be more trials with the test done in this pilot study. The most trials presented in any of the results is 3 trials. Also, as there was difficulty getting the stressor compounds to actually stress the cells, repeated trials may indicate a trend with the compounds and stressor compounds and could present results suggesting FAB and NMDA are not the appropriate stressors to use in this experiment. Exposing

the cells to another compound thought to induce neurotoxicity in the cultures or continuing stimulating the cells with FAB or NMDA could indicate the limitability of the drugs' neuroprotective effect. If more trials are done with either a new stressor or repeated trials using the same stressors already presented continue to show little to no neuroprotective affect from LM11A-31, Resveratrol, and Methylene Blue, this could disqualify these compounds as a viable treatment for AD.

The assays used to evaluate the neuroprotective ability of the drugs was limited to cell survival and microtubule intensity. While these tests are useful in a pilot study to assess the basic effects of adding a new combination treatment to combat neurotoxicity, there is room for further exploration. I hypothesized that the drugs Resveratrol, LM11A-31, and Methylene Blue would produce a stronger neuroprotective effect together against a neurotoxicity because they are thought to affect many of the same hallmarks and pathways of AD onset. However, my results did not support this hypothesis and yes that could have been because of the difficulty of getting the stressor compounds to actually stress the cells and produce neurotoxicity, but there could be a few other reasons why this happened and future studies are needed. I presented potential pathways in which each of the drugs may contribute to neuroprotection against stress induced by the hallmarks of AD (schematic 2). For instance, LM11A-31 and Resveratrol are suggested to modulate the neuronal death pathways induced by Aβ accumulation such as the Pi3K/AKT, GS3KB and AMPK pathways. Resveratrol and Methylene Blue are also suggested to affect the activity of caspases and oxidative stress pathways. While each of these drugs has a different way to modulate these pathways (i.e., LM11A-31 modulates the p75NTR while Resveratrol modulated the SIRT1 protein), primary literature indicates they all contribute to neuroprotection. I hypothesized that these drugs would interact and produce a positive effect, but I did not

measure the actual activity and interactions between these drugs. Future studies would include evaluating proteins expression levels of protein levels associated with the different pathways (i.e., SIRT 1, AMPK, GS3KB, and NAD). Whether these compounds individually or together produce a neuroprotective effect or not is it important to understand what is happening at the molecular level as to why the stimulations produce the results that they do. If more trials are done using the combination treatment strategies and eventually results suggesting a neuroprotective effect, we need to know why this is occurring by evaluating the different possible pathways. For instance, protein assays such as western blotting may be used to test if Resveratrol does effect SIRT1 activation or expression and if the other two compounds increase this effect and further promote neuroprotection. Overall, this pilot study sets up future studies to answer the how and why these drugs may be used to treat AD.

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