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Investigating the detrimental effects of mutant tau unable to bind to microtubules in mammalian COS-7 Cells

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Abstract

Neurofibrillary tangles of hyperphosphorylated tau are a fundamental hallmark of Alzheimer's disease. In this study, we investigate the behavior and impact of mutant tau with MTBD deletion is unable to bind to microtubules. Through use of DNA from mice and molecular cloning, we create plasmids with WT and mutant tau DNA that can then be expressed in COS-7 cells to study the behavior and effect of mutant tau in COS-7 cells, especially with regards to the microtubule network. Immunostaining of COS-7 cells expressing the mutant GFP-tau gene depicts inability to bind to microtubules, aggregate formation, and diffused masses within the cell. We conclude that if these mutant tau proteins were to be in a cell that has microtubules reliant on tau for stability, the mutant tau would negatively affect the dynamic instability of microtubules. To investigate the effect of mutant tau proteins on cell viability, drug treatments that negatively affect the dynamic instability of microtubules were used as a unit test to mirror the effect of mutant tau proteins. The results display that microtubules lacking dynamic instability cause cell death. Through the tests conducted in this study, we conclude that mutant tau in neurons would likely cause neurodegeneration.

Keywords
Alzheimer's, Hyperphosphorylated tau, Microtubules, Immunostaining and COS7.

1. Introduction
Alzheimer's disease is a neurodegenerative disease that currently affects around 6.5 million Americans. Alzheimer's disease is the most common cause of dementia, the progressive decline in cognition that affects memory, judgment, language, visuospatial abilities, and personality. The disease is associated with shrinkage of the cerebral cortex, expansion of fluid ventricles within the brain, and diminution of the hippocampus. These effects can be generalized to a more fundamental phenomena: neuron death, and thus Alzheimer's is called a neurodegenerative disease. The harm done by these effects to the brain is irreversible, as neurons do not divide, and thus loss of neurons is permanent. In addition to ultimately becoming fatal, Alzheimer's disease is especially devastating in that it alters the brain and by doing so alters the patient and fundamental aspects of their identity. This experiment examines the tau hypothesis of Alzheimer's disease by analyzing the effects of hyperphosphorylated tau on COS-7 cells.

1.1 Objectives
In this study, we investigate the effects of tau unable to bind to microtubules, i.e. hyperphosphorylated tau in neurons and mutant tau in this project, on cell viability to gain insight into how hyperphosphorylated tau impacts neurons and neuron viability. By doing so, we hope to gain more clarity on the cause(s) of Alzheimer's disease and thereby develop possible cures to the disease.

2. Literature Review
Despite the prevalence and devastating effects of Alzheimer's disease, there are no truly effective treatments that significantly alter the course of Alzheimer's disease. This is in part because the cause of Alzheimer's disease is not yet clear, and thus treatments are unable to directly eliminate the cause. Most Alzheimer's cases are idiopathic, i.e. pathologists are unable to diagnose the cause of the disease or understand why it has occurred in the patient (Lathuili`ere et al. #)

There are currently two main hypotheses theorizing the cause of Alzheimer's disease in patients: the β-amyloid hypothesis, and the tau hypothesis. The two of these hypotheses address the two fundamental pathological

hallmarks of Alzheimer's: amyloid plaques and neurofibrillary tangles, which have been shown to consist of tau in previous studies (Grundke-Iqbal et al. #). Failure in 2018 drug trials has tilt the scale towards the tau hypothesis (Kametani and Hasegawa #)}, the topic (Grundke-Iqbal et al. #)of interest for this project.

3. Methods

To test the effect of tau with mutations1 on the MTBD domain, we express mutant tau with microtubule binding domain (MTBD) deletion in mammalian COS-7 cells via transfection and compare the results with COS-7 cells transfected with wild-type tau from mice. We demonstrate that mutant tau with MTBD deletion is unable to bind to microtubules, forms aggregates, and thus would negatively affect microtubule dynamics in neuronal cells. An investigation with drug treatments which negatively affect the dynamic instability of micro- tubules shows this dynamicity to be crucial to cell viability. Our findings establish that tau, unable to bind to microtubules, i.e. mutant tau in this study and hyperphosphorylated tau in the neurons of Alzheimer's patients, can cause neurodegeneration.

We began with the extraction of tau gene from mice. PCR was run to amplify a particular segment of interest, tau gene, from the genomic cDNA extracted from mouse tail tissue. PCR was also used to create microtubule binding domain (MTBD) deletion mutations.

4. Results and Discussion

Our first result is a confirmation of whether or not PCR worked using gel electrophoresis: Figure 1 depicts the image of a test run where tau gene exon 9 of mouse tail genomic DNA was amplified with PCR to familiarize ourselves with lab equipment.

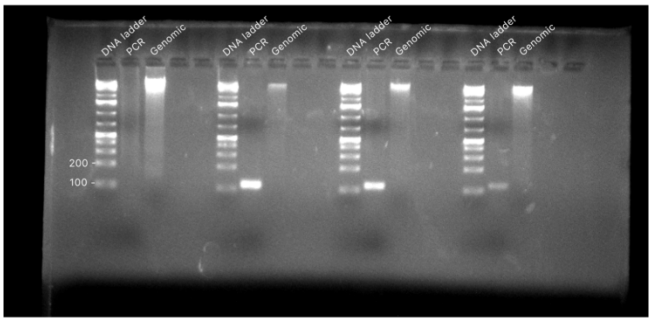

Figure 3.1. Gel electrophoresis of mouse genomic DNA and PCR sample of amplified tau exon 9

The gel electrophoresis run did not work for the second column, which contained a PCR sample. We then proceeded to rerun PCR two times, and the sample finally ran correctly in the third run, as seen in Figure 2.

Evaluating potential errors in the previous runs, we see that issues with the general setup of PCR or gel are unlikely, as the other PCR samples ran successfully on the gel. Thus, errors such as loading gel issue, gel over-run, incorrect light source during imaging etc. are not feasible sources of error for this case.

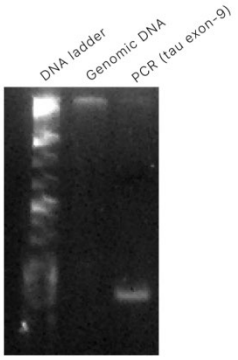

Figure 3.2. Successful PCR run with same genomic DNA as previously unsuccessful column 2 in 3.1

After the process of elimination, the most probable error is a low quantity of sample. The ge- nomic DNA extraction itself was successful, as the genomic DNA band ran nicely on the gel in every run. Therefore, the mistake likely lies in omitting DNA from the PCR sample before insertion into the PCR machine or an issue with diffusion when running the gel. Issues with proper pipetting were later found, where the pipet used for preparing PCR samples was being paired with the wrong tip, causing low to no amounts of particular ingredients in the PCR sample. When running the gel, the samples were also left to diffuse in agarose solution Figure 2. Successful PCR runs with the same genomic DNA as previously unsuccessful column 2 in 3.1 for quite long, especially in the second run, so that may explain the error in that iteration.

The next two results verify that the correct plasmids have been chosen, i.e. plasmids with the tau gene correctly inserted, and amplified via restriction enzyme digestion and gel elec-trophoresis. To amplify the prepared plasmids with wild-type and mutant tau for transfection, we trans- formed bacteria with the plasmids and then grew them on agar plates with antibiotic se- lection. The plasmids are conjugated with a gene for either kanamycin or ampicillin resistance; the plates indicate that both plasmids (wild-type and mutant) are conjugated with kanamycin resistance as successfully transformed bacteria survive on kanamycin agar plates but no bacteria survive on ampicillin agar plates, as pictured in Figure 3.

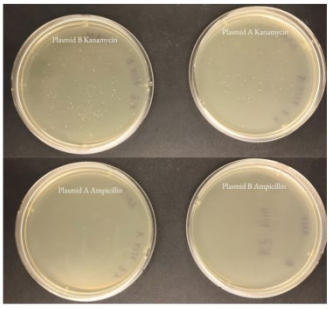

Figure 3.3. E. coli cultures of transformed bacteria after overnight incubation on agar plates with antibiotic selection

Bacterium that are able to grow on agar plates with kanamycin are selected for as the re- sistance to kanamycin indicates successful transformation. We calculate transformation efficiency to get a quantitative reflection of how many bacteria were successfully transformed. For the kanamycin agar plate with bacteria transformed with plasmid A, the transformation efficiency was around 370; for the kanamycin agar plate with bacterium transformed with plasmid B, around 725. 0 cultures were found in both ampicillin agar plates; the few dots on the sides of the plates and the one dot at the center of the ampicillin plasmid A plate are bubbles rather than colonies. Transformation efficiency was calculated as below:

$$\text{Transformation efficiency} = \frac{\text{No. colonies}}{\mu\text{g}}$$

$$= \frac{\text{No. colonies}}{0.1\frac{\mu\text{g}}{\mu\text{l}}(\text{plasmid sample DNA concentration} \times 2\mu\text{l of plasmid DNA sample used}}$$

$$= \text{No. colonies} \times 5.$$

Note that the plasmids are labeled as A and B until we verify their identity with restriction enzyme digestion in a later step.

We then collected a few colonies from the plate and put them in a tube with LB growth medium for further amplification and ease of plasmid DNA extraction. After overnight incubation, the tubes appear cloudy and we use Mini-prep kits to isolate and purify plasmid DNA from the E. coli. After isolation and purification, we use DNA spectrometry to evaluate the purity of the plasmid DNA isolated (i.e. amount of protein etc. contamination) and to find the concentration of plasmid DNA in our isolated plasmid solution.

$$\text{Concentration of plasmid DNA in plasmid A solution} = 0.237\frac{\mu\text{g}}{\mu\text{l}}$$

$$\text{OD260 / OD280 of plasmid A solution} = 1.8$$

$$\text{Concentration of plasmid DNA in plasmid ABsolution} = 0.270\frac{\mu\text{g}}{\mu\text{l}}$$

$$\text{OD260 / OD280 of plasmid B solution} = 1.75$$

OD260 / OD280 is a ratio of DNA to protein: a pure DNA sample will yield an OD260 / OD280 ratio of approximately 1.8. A lower ratio indicates some protein contamination. We can observe that the plasmid A solution was very successfully purified, whilst the plasmid B solution contains some proteins. However, an OD260 / OD280 ratio of 1.75 is close enough to 1.8, so we do not repeat the purification.

We then performed restriction enzyme digestion mapping on the plasmid solutions to verify the identity of plasmid A and B (wild-type tau or mutant). Plasmids were cut with Nhe1, Kpn1, and both. Plasmids were incubated in the specified restriction enzyme(s) for 45 minutes then frozen to preserve the plasmids for gel electrophoresis later. We now review the gel electrophoresis results, which are imaged in Figure 4.

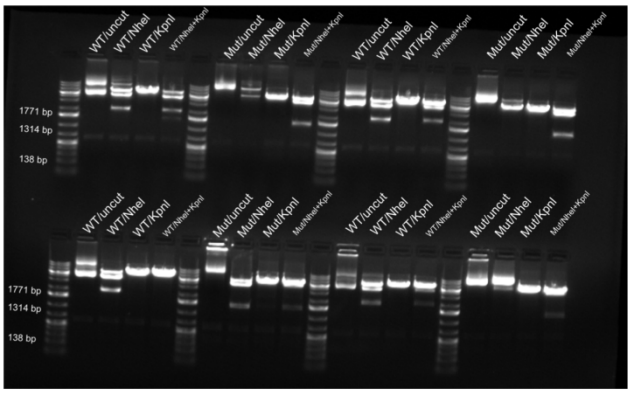

Figure 3.4. Restriction enzyme digestion results of 4 samples

Comparing our gel electrophoresis results with the known gel electrophoresis results for wild- type and mutant plasmids cut with the same enzymes (Figure 5), we see that plasmid A is conjugated with wild-type tau gene and plasmid B is conjugated with mutant tau gene. Note that this restriction enzyme digestion mapping is able to distinguish between the two plasmids because the mutant tau gene contains a MTBD deletion, which deleted a Nhe1

restriction site, and thus wild-type tau gene plasmids are cut into two pieces when incubated with Nhe1 but mutant plasmids are only cut into one piece.

After verifying the identity of the plasmids and amplifying them, we move onto transfection results. To transfect COS-7 cells with wild-type and mutant tau plasmids, we used Turbofect and in- cubated the cells for 24 hours. We then used GFP imaging to calculate transfection efficiency as the plasmids were conjugated with GFP. Figures 6 and 7 depicts the images taken of the cells: COS-7 cells that fluoresce green are expressing GFP, and are thus successfully wild-type tau and mutant tau

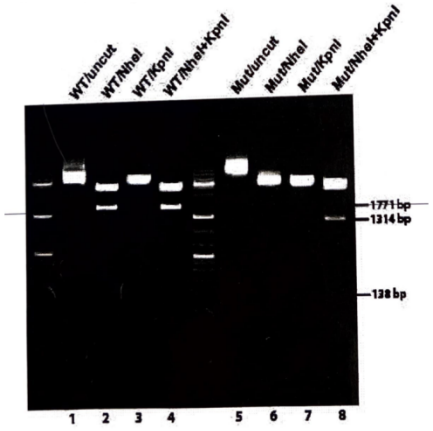

Figure 3.5. Known restriction enzyme digestion mapping of plasmids conjugated with wild-type tau and mutant tau

Transfection efficiency for COS-7 group transfected with wild-type plasmid (3.6) ≈ 50 %
Transfection efficiency for COS-7 group transfected with mutant plasmid (3.7) ≈ 35 %

The transfection efficiency for both COS-7 groups is sufficient for immunostaining, which we conduct in the next step. Possible reasons a higher transfection efficiency was not obtained include low concentration of plasmids, plasmids that are not of ideal purity, and a relatively short wait time. We incubated the cells for 24 hours but we can theoretically wait up to 72 hours, waiting longer may yield a higher transfection efficiency.

4.1 Immunostaining transfected COS-7 cells

After confirming that a decent amount of COS-7 cells have been successfully transfected, we move on to immunostaining. We now evaluate the results of immunostaining the transfected COS-7 cells.

Figures 8 and 9 display the immunostained COS-7 cells magnified at 40x. The colocalization of green and red fluorescence demonstrates a close relationship and interaction between tau and microtubules, which, based on the knowledge of tau as a MAP protein with a microtubule binding domain, depicts that the tau transfected into the cell has successfully bound to the microtubule network in COS-7 cells. We can infer from the immunostaining that if wild-type tau protein were to be in neurons, it would be able to fulfill its role of stabilizing the internal skeleton of nerve cells ([GNH17]).

Contrarily, the immunostaining images obtained of COS-7 cells transfected with mutant tau plasmid display mutant tau unable to bind to microtubules, seen through the lack of colocalization between the two proteins in Figures 10 and 11. Instead, the mutant tau protein can be observed forming aggregates in the images and appearing diffused rather than taking on the fibrous appearance of microtubules as wild-type tau protein does.

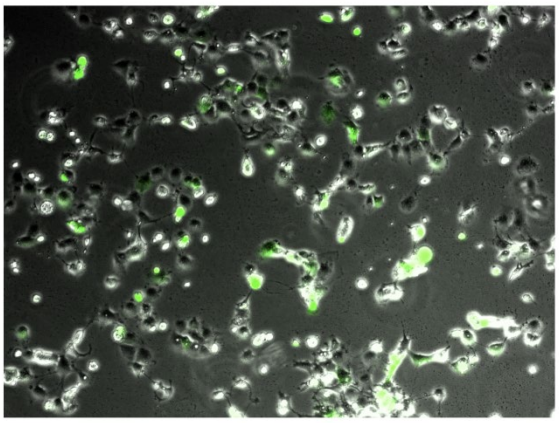

Figure 3.6. GFP imaging of COS-7 cells transfected with wild-type tau plasmid on coverplates

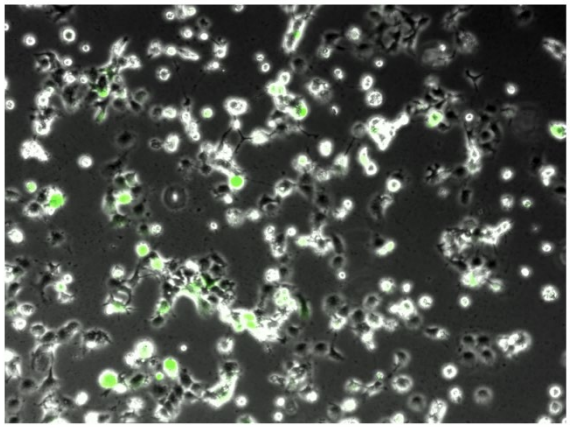

Figure 3.7. GFP imaging of COS-7 cells transfected with mutant tau plasmid on coverplates

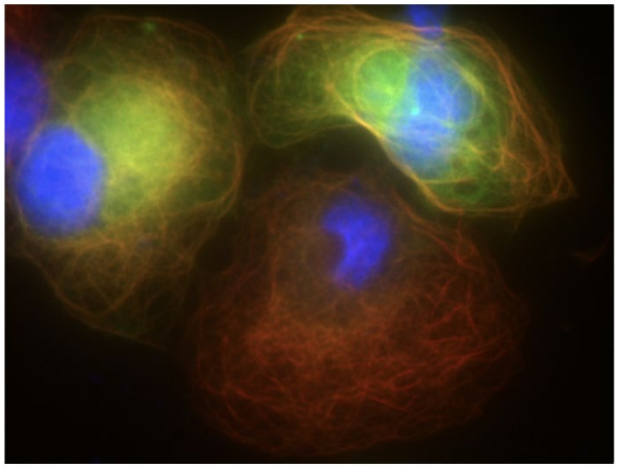

Figure 3.8. Immunostaining for GFP protein on tau and α-tubulin in COS-7 cells transfected with wild-type plasmid. Image 1.

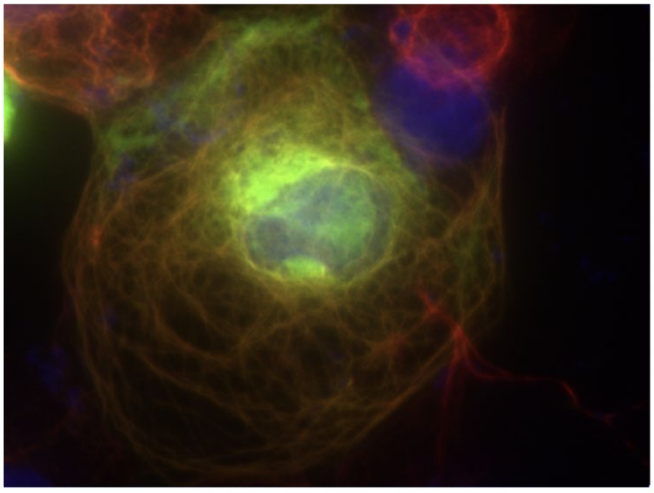

Figure 3.9. Immunostaining for GFP protein on tau and α-tubulin in COS-7 cells transfected with wild-type plasmid. Image 2.

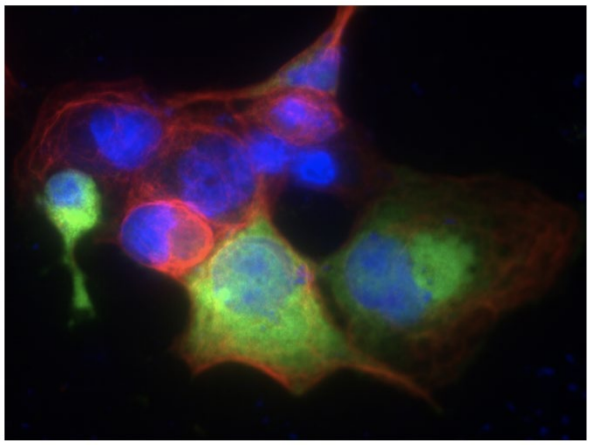

Figure 3.10. Immunostaining for GFP protein on tau and α-tubulin in COS-7 cells transfected with mutant plasmid. Image 1

To focus exclusively on tau protein's behavior after transfection into the COS-7 cells, we compare the FITC channel in isolation without the overlay of TRITC and DAPI channels. Figure 12 provides a side by side comparison of the FITC channel for COS-7 cells transfected with wild-type tau protein (left) vs. mutant tau protein (right). Whilst the wild-type tau protein shows a clear adherence to the fibrous microtubular structures within the COS- 7 cells indicating successful binding to microtubules, the mutant tau protein is scattered throughout the cell and in aggregates.

We conclude from these series of microscope results that the mutant tau protein with MTBD deletion is largely unable to bind to microtubules and thereby forms diffused aggregates of tau in the cell instead. However, although mutant tau protein forms aggregates throughout the COS-7 cell, the microtubular network of the cell remains largely unaffected, as seen in Figure 11 where the red fibers do not significantly differ from the fibers in the normal, wild-type sample except that they lack the colocalization of tau. This result can be explained by the fact that COS-7 cells are not natively dependent on tau protein for microtubule stability: COS-7 cells normally produce little to no tau and thus their microtubular network has other proteins to depend on for microtubule stability. Therefore, mutant tau does not have a significant effect on the microtubule network of COS-7 cells. However, the behavior of mutant tau in COS-7 cells, i.e. the inability to bind to microtubules, disassociation from microtubules, and formation of

aggregates, displays that mutant tau would be detrimental to cells that have microtubules reliant on tau's stability support for structure and function, such as neurons.

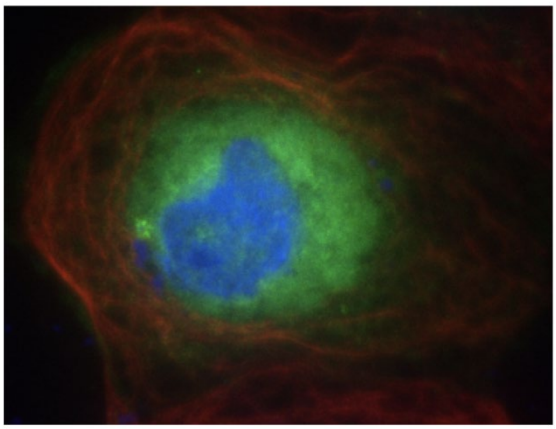

Figure 3.11. Immunostaining for GFP protein on tau and α-tubulin in COS-7 cells transfected with mutant plasmid. Image 2

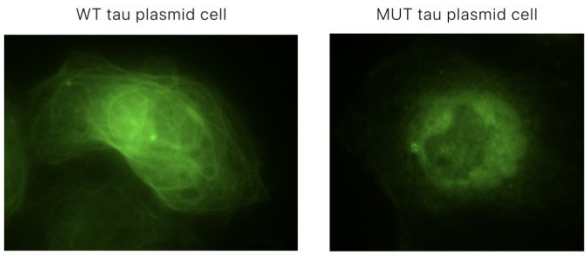

Figure 3.12. Comparing tau staining [anti-GFP] in COS-7 cells transfected with wild-type and mutant plasmid (MTBD deletion)

Tau protein is native to neurons and helps stabilize the microtubules. Our results display that if tau were to act as mutant tau does in COS-7 cells, neuron microtubule stability would be significantly impacted in that instead of serving as support, the tau proteins aggregate and form diffused masses instead. In addition, hyperphosphorylated tau is known to be unable to bind to microtubules as mutant tau does (Lathuili`ere et al. #)

4.2 Nocodazole and COS-7 cells

We then investigated the effects of this loss in microtubule stability tau, unable to bind to microtubules would induce in neurons via using drug treatments that cause irregular mi- crotubule stability in COS-7 cells. The two drugs used are Taxol and Nocodazole. Taxol binds to microtubules and stabilizes them against depolymerization, disrupting normal mitotic spindle formation and thus preventing cell division. Nocodazole is an antineoplastic agent, which exerts its effect in cells by interfering with the polymerization of microtubules; cells treated with Nocodazole undergo microtubule network depolymerization. Both drugs affect microtubule dynamics: Taxol excessively stabilizes microtubules; Nocodazole excessively destabilizes microtubules.

Prior to running a viability assay, immunostaining was used to visualize the effects of Taxol and Nocodazole on microtubules. The results are shown in Figures 13 and 14. Alpha- tubulin in microtubules was stained for with primary antibody mouse 12G10 anti-alpha- tubulin and secondary antibody goat anti-mouse conjugated to Alexa 594. As expected, Taxol induced over-polymerization of microtubules (see Figure 13) due to its stabilization of microtubule polymers and protection of microtubules from disassembly; Nocodazole dis- assembled microtubules (see Figure 1)

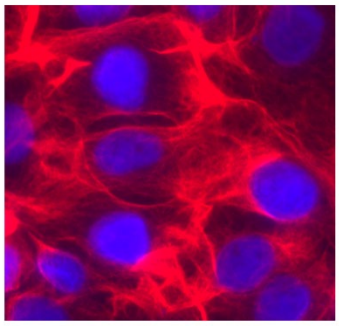

Figure 3.13. Taxol alpha-tubulin immunostaining [Red is anti-alpha-tubulin; blue is DAPI]

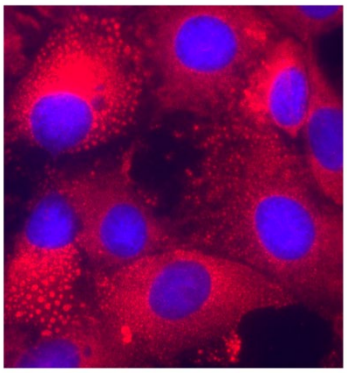

Figure 3.14. Nocodazole alpha-tubulin immunostaining [Red is anti-alpha-tubulin; blue is DAPI]

We now examine the effects of irregular microtubule stability and the effects on cell viability via a viability assay. Tau in neurons stabilizes microtubules, so neurons with hyperphosphorylated tau that is unable to bind to microtubules experience an effect similar to Nocodazole in cells, except less extreme.

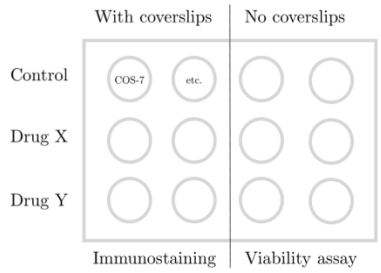

Figure 3.15. Viability assay well plate set up

A viability assay was set up with 4 groups consisting of Taxol diluted with DMSO, Nocodazole diluted with DMSO, DMSO, and H2O2. Because Taxol and Nocodazole were diluted with DMSO prior to administration to the COS-7 cells, DMSO was set up as a negative control to confirm that DMSO is not the variable affecting cell viability. Results from the viability assay DMSO well indeed confirms this: DMSO did not have significant effects on cell viability. H2O2 was used as positive control for additional control group.

The viability assay was set up in a well plate in accordance to Figure 15. Wells with cover slips were used for the immunostaining conducted above. Wells without cover slips were used for the viability assay.

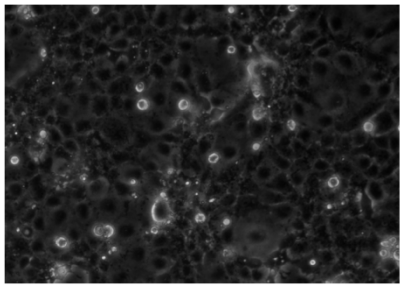

Figure 3.16. Phase-contrast image of DMSO group. Cells are mostly alive and attached to the well plate with a dark appearance.

For the viability assay, 10 μL of Nocodazole and Taxol were added to the Drug X and Drug Y wells without coverslips [Drug X being Nocodazole and Drug Y being Taxol]. The cells were then incubated overnight, along with the control well containing DMSO. 50 μL H2O2 was added to the positive control well for 30 min. before counting the number of alive/dead cells with Trypan blue and a hemocytometer.

Prior to conducting a count with the hemocytometer, phase-contrast images were taken of the wells for a qualitative estimate of the number of dead cells. Live cells appear darker and are attached to the well as the COS-7 cells used adhere to the surface which they grow on. Dead cells appear lighter in color and are floating in the well, as they have lost the ability to attach to the well. Figures 16, 17, 18, and 19 display the phase-contrast images of the DMSO well, Taxol well, Nocodazole well, and H2O2 well respectively.

Analyzing the phase-contrast images of of all 4 groups [DMSO, Taxol, Nocodazole, H2O2], we see that the qualitative estimate of our results matches what we expect: the DMSO group holds the most number of cells that are alive, both Taxol and Nocodazole reduced cell viability, and H2O2 caused all COS-7 cells in the well to die.

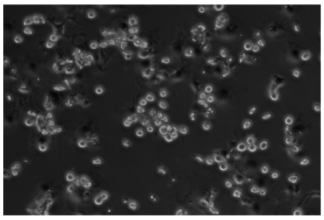

Figure 3.17. Phase-contrast image of Taxol group. More cells are dead than the DMSO control group, as expected.

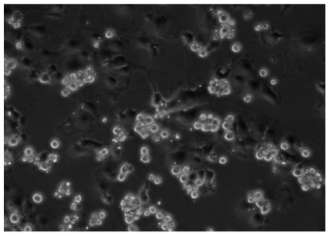

Figure 3.18. Phase-contrast image of Nocodazole group. More cells are dead than the DMSO control group, as expected.

To obtain a more accurate distinction between the number of cells alive after Taxol administration vs. Nocodazole administration, a quantitative estimate was obtained with a Trypan blue test. For each group, the cells in the well plates were collected and suspended in Trypan blue. Trypan blue stains dead cells but does not stain cells that are alive, as dead cells lose the intact selective cell membranes which prevent the absorbance of the stain in viable cells.

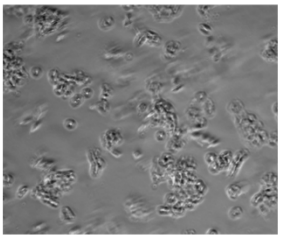

Figure 3.19. Phase-contrast image of H_2O_2 group. All the cells are dead, as depicted by the white halo around the cells. In addition, this image was taken when the well plate was being shaken, and depicts that the cells are all floating as the blur results from the cells moving around in the solution in different directions due to no longer being attached to the well plate.

Therefore, after suspending the cell solution in Trypan blue and viewing it under a microscope, dead cells will be stained blue whilst alive cells will be white.

To count the number of viable and dead cells in the solution with Trypan blue, 10 μL samples of the solution were used to fill a hemocytometer. The number of dead and alive cells were calculated via counting the number in one square times 25:

$$\text{Count of alive} = \text{Count of alive cells in 1 square} \times 25$$

$$\text{Count of dead} = \text{Count of dead cells in 1 square} \times 25$$

As a note, when counting the dead cells, some cells were fragmented. The convention used in counting for this paper counted 2 fragments as 1 cell.

$$\%\ \text{alive} = \frac{\text{Count of alive}}{\text{Count of alive} + \text{Count of dead}}$$

$$\%\ \text{dead} = \frac{\text{Count of dead}}{\text{Count of alive} + \text{Count of dead}}$$

Table 1 displays the relevant statistics for each group, calculated with the formulas above.

	Count of alive cells	Count of dead cells	% alive	% dead
DMSO	13	3	81.25	18.75
Nocodazole	1	9	10	90
Taxol	3	7	30	70
H_2O_2	0	11	0	100

Table 1. Trypan blue viability assay results

Figure 20 gives the cells counted. Because the cells were left in Trypan blue solution for longer than usual, alive cells are also tainted slightly blue, but close examination shows their cell membranes to be intact. Figure 21 displays the difference for reference of this paper's procedure.

Figure 22 graphs the results recorded in Table 1 as a visualization of the data.

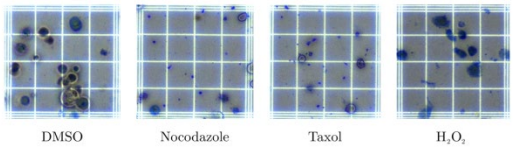

Figure 3.20. Hemocytometer cells for each group

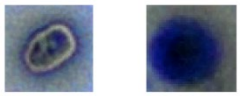

Figure 3.21. Alive vs. dead cells stained with Trypan blue

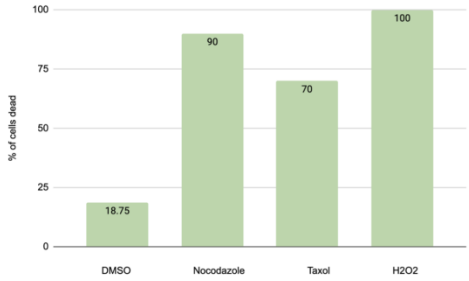

Figure 3.22. Graph of Trypan blue staining results

The results indicate that a loss of microtubule stability is detrimental to cell viability. Nocodazole mimics the instability and thereafter disassembly microtubules would experience if tau, a microtubule associated protein that contributes to stability, where to disassociate from microtubules as tau does when it becomes hyperphosphorylated. Thus, we conclude that hyperphosphorylated tau would likely decrease neuron viability.

5. Conclusion

The results of this study indicate that tau unable to bind to microtubules will induce a loss of microtubule stability in neurons and thereby neuronal death, providing support for the tau hypothesis of Alzheimer's disease. Transfection results of mutant tau show tau aggregates and disassociation from microtubules and drug treatment of COS-7 cells with Nocodazole show unstable microtubules to induce cell death. Further studies may explore a more direct relation between mutant tau and neurons via transfecting neurons rather than COS-7 cells. The tau hypothesis may also be further tested through examining if treatments that prevent tau hyperphosphorylation or increase amounts of normal tau alleviate Alzheimer's symptoms and/or progression.

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Biography

Kaitlyn Zhang is a student at Stanford Online High School. She has earned awards in robotics competitions, as well as computation and business challenges such as Technovation. Kaitlyn's research interests include neurology and Alzheimer's disease and their impact on the current world.