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VALIDATION OF PHARMACEUTICALS LEADING TO THE REDUCTION OF INCLUSION OF TAU EXON 10

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VALIDATION OF PHARMACEUTICALS LEADING TO THE REDUCTION OF INCLUSION OF TAU EXON 10

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by

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ABSTRACT

Tau is a protein normally associated with the assembly of microtubules, but it tends to hyperphosphorylate leading to its aggregation which is a major cause of several neurodegenerative disorders, termed tauopathies that includes Alzheimer's disease. Alternative splicing of exon 10 in the *tau* gene has also been suggested to play an important role in the pathogenesis of tauopathies. When tau exon 10 is included in the tau protein, neurofibrillary tangles (tau aggregates) are more prevalent. To search for potential treatments for tauopathies, our laboratory previously developed a luciferase reporter assay to screen compounds that decrease tau exon 10 inclusion and identified five molecules from 1,040 FDA approved drugs for initial luciferase activity. The goal of our project was to further characterize the key five compounds identified in the original screen using the luciferase assay and RT-PCR. We demonstrated that all five compounds tested produced statistically significant increases in luciferase activity relative to DMSO control at 5 μM concentrations, validating the original screening.

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ACKNOWLEDGEMENTS

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BACKGROUND

Alzheimer's Disease

Alzheimer's disease (AD) is a progressive neurodegenerative disorder which predominantly affects the elderly. AD involves the destruction of neurons which control memory, thought and language. Currently in the United States, there are over five million people living with the disease. There are two types of AD: Early-onset, and late-onset. Late-onset cases of AD are primarily seen in the 65 years and over category. Early-onset AD, sometimes associated with genetic predisposition, is seen in patients under the age of 65.

There are seven stages of AD, increasing from no impairment to very severe decline. A patient who is diagnosed with AD could fall within any of these seven stages at any given time. The disease also advances at different rates in different patients. Some people end up only living a few years with AD, whereas others can live with the disease for as long as twenty years (Alzheimer's Association, 2007).

Symptoms

Several symptoms can be observed in an AD patient. Some of the most prevalent symptoms are memory loss, disorientation to time and place, changes in mood and behavior, problems with language, and finally changes in personality. Some of these symptoms are synonymous with the normal aging process such as memory loss, but AD patients experience these symptoms more frequently.

Alzheimer's Disease Risk Factors

Certain risk factors have been observed in AD patients. The most obvious factor at the present time is age. As the brain continues to function, some of the proteins build up and cause neuronal cell death which results in the loss of brain function. Another risk factor is family history; however, in only five percent of cases is true familial AD observed. Certain abnormal genes, if inherited, will definitely cause the disease. For example, certain mutations found in PS1, PS2 or APP genes can cause AD. It is significant that mutations in each of these three genes have one thing in common, they each increase the formation of highly neurotoxic Aβ peptide from amyloid precursor protein (APP). The "amyloid cascade hypothesis" proposes a key role for neurotoxic Aβ in the formation of senile plaques and AD, but this mechanism is not the topic of this MQP, so will not be discussed in detail here.

In addition to genes that cause AD, some genes just increase the risk of developing AD. One such gene is the apoliprotein E-e4 (APOE-e4) gene. There are three common forms of the APOE gene, and the e4 form increases the risk of developing AD. Normally this gene is responsible for the creation of a protein that carries cholesterol in the blood stream, but when a person inherits either one or two copies of APOE-e4 they are more likely to develop AD. The APOE-e4 gene also accelerates the formation of highly neurotoxic amyloid-beta protein from amyloid precurson protein.

The final two risk factors that are avoidable are head trauma and unhealthy hearts. It has been proven that a healthy heart can keep the brain healthy for currently unknown reasons; however one hypothesis is that a healthy heart increases blood flow to the brain (Alzheimer's Association, 2007).

Current Treatments

Since AD is such a prevalent disease, the United States government has poured money into research that could help treat the disease. Right now the current treatments only slow down the progression of the disease, they don't stop it (Alzheimer's Association, 2007). And they treat only symptoms, not the underlying neurodegenerative cause. Five drugs are currently FDA approved for treating AD (dates of FDA approval and alternative names in parentheses): Tacrine (1993 Cognex®), Donepezil hydrochloride (1996, Aricept®), Rivastigmine (2000, Exelon®), Galantamine hydrobromide (2001, Razadyne™, formerly called Reminyl®), Memantine HCI (2003, NamendaTM). Each of these drugs blocks acetylcholine esterase, the enzyme that degrades acetylcholine, thereby increasing acetylcholine neurotransmitter in syapses. Vitamin E treatment also may slow down the disease, but can not completely stop the progression, or repair the damage that has already been done (Alzheimer's Association, 2007).

Alzheimer's Disease Sociology

Since the treatments can't stop the disease, patients are forced into nursing homes or forced to hire help faster than those people without the disease, and as such it is costing these people thousands of more dollars per year (Figure-1) (Alzheimer's Association, 2007).

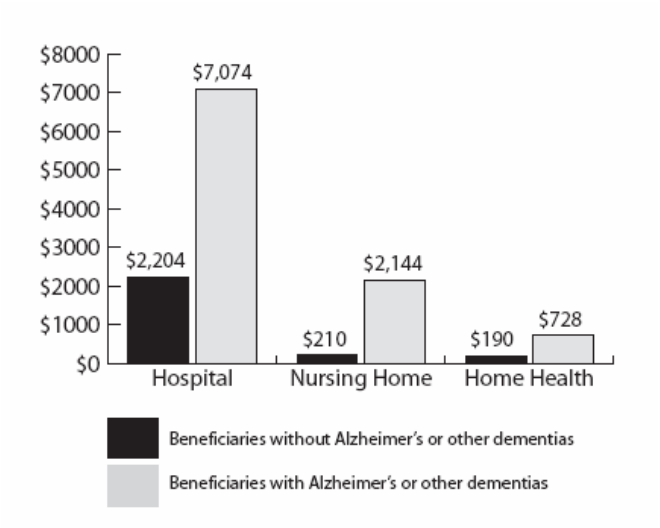

Figure 1: The High Cost of Alzheimer's Disease. Comparison of average medicare costs for hospital, nursing home, and home health care for beneficiaries 65+ with Alzheimer's Disease and other dementias (light gray histobars) as compared with other beneficiaries (dark histobars) (Alzheimer's Association, 2007).

Diagnosis

Perhaps one of the biggest problems with AD is there is no definitive test that determines whether a patient has the disease. In order to diagnose the disease, doctors perform multiple memory tests, such as the mini-mental state examination (MMSE). They also use blood tests to determine if there are any infections or other conditions that could be causing the memory problems. Finally, doctors can use brain imaging techniques such as an MRI or PET scan to try and see structural and functional changes that might have occurred. Figure-2 shows a PET scan of a normal brain (left panel) compared to an Alzheimer's patient's brain (right panel) (Alzheimer's Association, 2007) illustrating the lowered brain activity associated with AD.

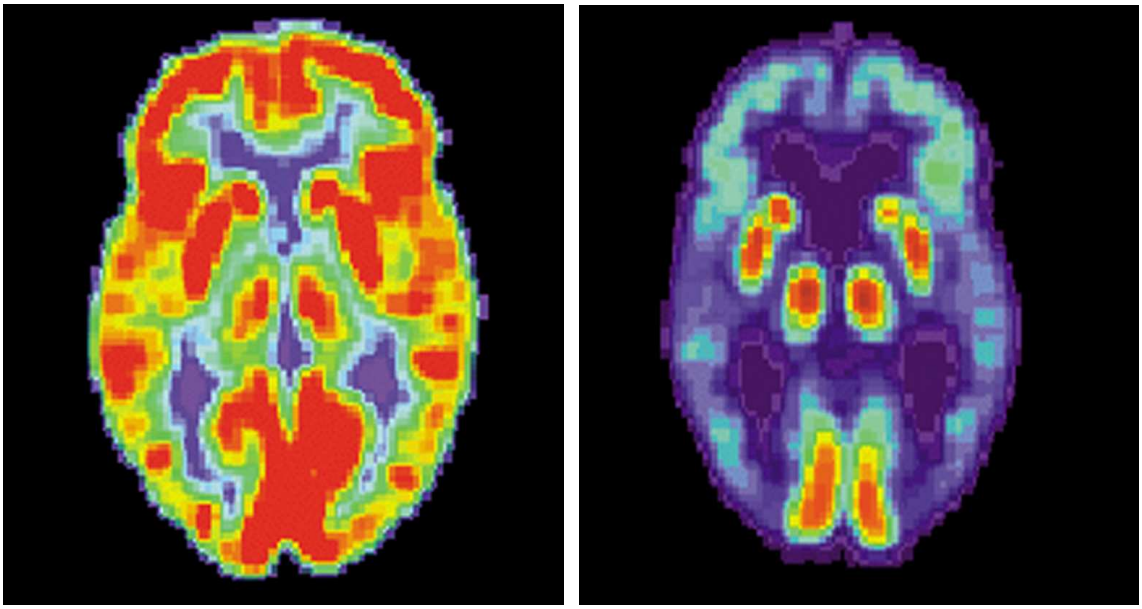

Figure 2: PET Scan Comparison of Normal and Alzheimer's Brains. On the left is a PET scan of a normal brain. Note that there is lots of red color (usually denoting labeled glucose uptake) that indicates healthy brain function. On the right is a PET scan of an Alzheimer's disease patient. Note the lack of red color indicating less brain activity (National Institute on Aging, 2006).

AD is difficult to positively diagnose prior to death. Recently some scientists have claimed to develop magnetic resonance imaging (MRI) assays that detect senile plaques in patients brains. And other scientists claim that the quantization of Aβ released into the cerebrospinal fluid (CSF) is an accurate measure of AD in progress. But in the end, the only way that AD can be truly diagnosed is after death during an autopsy. When medical examiners analyze slices of the brain, they will see the tell tale amyloid plaques caused by the build up of amyloid-β and the neurofibrillary tangles which are caused by the build up of tau protein in the brain.

Alzheimer's Disease Mechanism

The cellular causes of neurodegeneration in AD are currently unknown, however much research has focused on two hallmark features of the disease, extracellular senile plaques (composed of amyloid) and intracellular neurofibillary tangles (composed of Tau) (Goedert et al., 2006). Figure 3 shows a summary diagram of some of the key events that are currently thought to participate in neuronal apoptosis, including the production of neurotoxic Aβ peptide (yellow box in the diagram) and Tau hyperphosphorylation (diagram lower center). For years scientists have argued over whether Tau or Amyloid are the direct causes of AD, and whether one or both are disease side effects, but most current models of the disease argue both Aβ buildup and tau hyperphosphorylation are strong cellular causes. Because this MQP focused on Tau, the remainder of the discussion will focus on Tau not Amyloid.

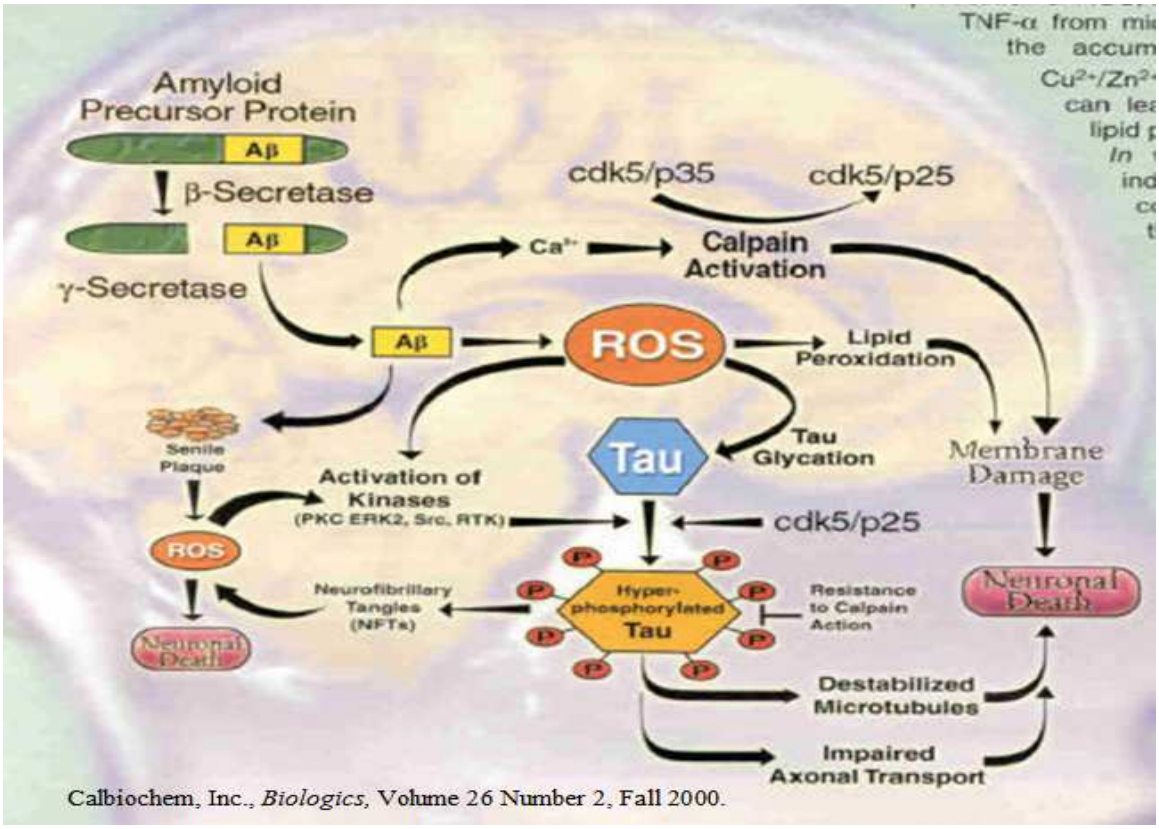

Figure 3. Diagram of Alzheimer's Disease Mechanism. Note the central role proposed for both Aβ peptide (yellow box) and Tau hyperphosphorylation (lower center). (Calbiochem, 2000).

Tau Protein

Tau protein is a highly soluble microtubule-associated protein (MAP) that binds to tubulin to stabilize microtubules and promote tubulin assembly into microtubules. Tau protein is a short rod-like molecule that forms cross bridges between microtubules *in vitro*. For the most part, the tau protein is responsible for the polymerization of tubulin which leads to better stability and assembly in neuronal process extension (Weingarten et al., 1975).

Tau Phosphorylation

There are two ways that tau is able to control microtubule assembly: phosphorylation and isoforms (Drubin et al., 1986). The phosphorylation of tau is regulated and performed by several kinases. Phosphorylation by these kinases is essential for the posttranslational modifications of the tau protein. There are two specific types of kinases that regulate tau: proline and non-proline directed protein kinases. The proline directed protein kinases (PDPK) phosphorylate tau through modified serine-proline or threonine-proline tau motifs. The three main PDPKs are tau kinase I, cdk5, and stress kinases. The non-proline directed protein kinases (NPDPK) phosphorylate tau through serine or threonine residues that are not followed by proline. The main NPDPKs are cyclic-AMP dependent kinase, Ca^{2+}/calmodulin dependent kinase, protein kinase C, and microtubule affinity regulating kinase.

Tau Domains

Ultimately, phosphorylation of tau allows for different binding sites (Figure-4, domains m1, m2, m3, m4) where the microtubule can be bound and moved to create a more stable structure, and the same holds true for the tau isoforms (Gómez-Ramos et al., 2004).

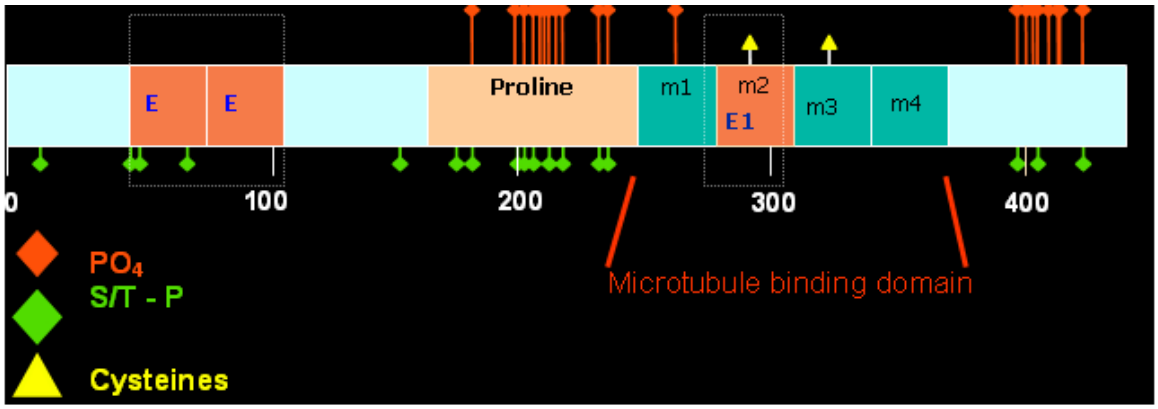

Figure 4: Tau Protein Domains. The diagram illustrates where phosphates are added (red), where the serine/threonine proline kinases can bind (green), where the microtubule binding domains are (m1, m2, m3, m4), and where each of the alternatively spliced exons (exons 2, 3 and 10) are (orange E boxes) (Kuret, 2006).

One of the major structural characteristics of the tau protein is the presence of a four carboxyl-terminal tandem repeat sequences of 31 or 32 amino acids that are encoded by exon 9, 10, 11, and 12. The four carboxyl-terminal tandem repeat sequence is used to bind and stabilize microtubules. As seen in figure 3, there is a proline rich region in the middle of the protein. Without this proline rich region, the tau protein will not bind to microtubules. The microtubule binding region that is seen in figure 3 contains a repeated domain, and without that repeated domain, tau will not bind to microtubules either. There is also a neutral domain in the N-terminal region, and a region in the first half of the C-terminal tail of the protein that when disrupted cause the protein to become less efficient at microtubule bundling.

Tau mRNA is expressed mainly in neurons and in some oligodendrocytes. Within nerve cells, the axon holds most of the tau protein. Tau does not seem to be an essential protein in mice because when its gene is inactivated, there are no adverse affects on that phenotype. Only the amount of microtubules in some small-caliber axons is reduced (Spillantini et al., 1998).

Tau Isoforms

The human *tau* gene contains sixteen exons, and encodes a full-length 441 amino-acid protein. The human gene is also more than 100 kilobases long and has been mapped to chromosome 17q21. The first exon is the promoter, and exons 4A, 6 and 8 are not present in human mRNA. Exons 2, 3 and 10 are alternatively spliced which create six different mRNAs that have been observed. These six mRNAs are responsible for the six different isoforms of the tau protein. Exons 2 and 3 can be found in the N-terminal region of the protein, and exon 10 can be found in the C-terminal end of the protein. The adult tau proteins can range anywhere from 352 to 441 amino-acids long. The tau protein isoforms that have been found in fetuses exclude exons 2, 3 and 10. From species to species, there are great differences in the tau protein formation. For instance, in rats there are only three isoforms (Yu et al., 2004). These regions as well as all six isoforms of the protein can be seen in Figure 5 (Kanai et al., 1992).

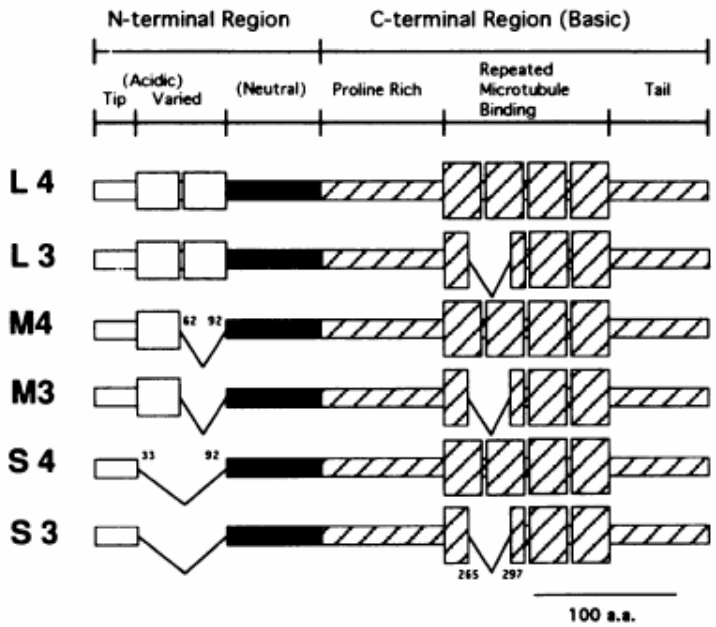

Figure 5: Tau Isoforms. This figure shows all six isoforms of the tau protein including the composition of the N-terminal region and C-terminal tail for all forms (Kanai et al., 1992).

Tau in Alzheimer's Disease

Neurodegenerative disorders commonly known as tauopathies are caused by the irregular expression or malfunction of tau protein, the protein normally recognized to bind and assemble microtubules. AD is one of the more common tauopathies. Many neurodegenerative disorders such as frontotemporal dementia and parkinsonism are linked to chromosome 17 (FTDP-17). AD is linked to chromosomes 21 (amyloid precursor protein), 14 (aopE), and 17 (tau). All these disorders possess the familiar pathological characteristic of the development of insoluble intraneuronal aggregates compiled of hyperphosphorylated tau proteins that develop neurofibrillary tangles. Neurofibrillary degeneration, also known molecularly as tauopathies, affects the cortical and subcortical regions of the brain. Neurofibrillary degeneration is characterized by the presence of intraneuronal inclusions that are found in the brain. These inclusions react with anti-tau antibodies and form bundles that are called neurofibrillary tangles. The theory that tau genotype could affect the progress of neurodegenerative diseases came from the detection of a polymorphic marker of the *tau* gene that is over expressed in progressive supranuclear palsy. The tau protein and tauopathies are genetically related since linkage analysis of frontotemporal dementia and parkinsonism and many other neurodegenerative diseases were mapped to the same region as the *tau* gene on Chromosome 17 (Schraen-Maschke et al, 2004).

Many mutations were discovered in the *tau* gene from patients with such neurodegenerative diseases. A minimum of 29 discrete pathogenic mutations of the *tau*

gene were recognized in many families with frontotemporal dementia (FTDP-17). A majority of these frontotemporal dementia Tau mutations were found to be in the coding region or close in proximity to the splicing donor site of intron 10. Exonic mutations, found in close proximity to the microtubule-binding domain, alter the ability of tau to encourage proper microtubule assembly. Intronic, silent, and several missense mutations have been demonstrated to influence mRNA splicing of exon 10 (Umeda et al, 2004). Furthermore, it has been demonstrated that a single group of mutations in coding sequences is able to modify the capacity of tau to interact with microtubules. Other missense mutations found in the coding region do not alter the affinity of tau protein to microtubules. These mutations, as well as mutations in introns, affect the splicing of exon 10. Two major Tau isoforms, 4R Tau (4 repeats) and 3R Tau (3 repeats) are created as a result of alternative splicing of tau exon 10. The 3R/4R Tau ratio, normally 1:1, is altered with a shift towards a higher production of the 4R protein. Further investigation of *tau* mRNA and proteins demonstrated that tau protein with exon 10 inclusion is in excess in patients with frontotemporal dementia and progressive supranuclear palsy which shows that abnormal splicing of exon 10 exists in other neurodegenerative diseases besides frontotemporal dementia (Yu et al, 2004). No tau mutations have yet been mapped to AD.

The role of the *tau* gene in the pathogenesis of tauopathies such as FTDP-17 and progressive supranuclear palsy was not understood until mutations of the gene were discovered. The fact that a cluster of mutations affects the splicing of exon 10 introduces the hypothesis that polymorphisms within introns 9 or 10 that influence *tau* splicing could cause tauopathies. It is known that the splicing of exon 10 is developmentally

controlled. In fact, infants only express exon 10 excluded mRNA. Exon 10 inclusion augments with age, with the normal ratio of exclusion to inclusion of exon 10 being 1:1. Many neurodegenerative diseases are dependent upon age. Therefore it is feasible that variations in the splicing of exon 10 have lasting effects on the aggregation of tau proteins. As a result, the detection of drugs or naturally occuring molecules that alter *tau* splicing could be beneficial for treatment of FTDP-17, PSP, and other neurodegenerative diseases (Yu et al, 2004).

Consequently, attempts at discovering compounds that hinder the construction of Tau filaments and the hyperphosphorylation of tau proteins have been made. The discovery of mutations that support the inclusion into *tau* mRNA of exon 10 in several FTDP-17 patients offers support that exon 10 inclusion may have a role in other tauopathies.

Design of Assays to Detect Alternative Splicing in SMN and Tau

Dr. Jianhua Zhou's lab (UMass Medical School, Worcester) has established a cell-based screen for small compounds that possess the ability to encourage the inclusion of exon 7 in the survival motor neuron (SMN) gene, responsible for Spinal Muscular Atrophy (SMA). This system was organized so that compounds that increase luciferase activity would be the ones that heightened the inclusion of exon 7 into SMN mRNA. The system was constructed using a mini-gene with a minimal length exon 7 with a luciferase reporter fused to exon 8. Therefore, when exon 7 was introduced into the mRNA, luciferase would be within a reading frame, whereas in the absence of exon 7 the mRNA would lack the luciferase activity (Zhang et al, 2001).

Dr. Zhou's lab has also used similar technology to construct an assay to detect *tau* exon 10 exclusion. This system was assembled after identifying the fact that a minimal distance of intron 10 is necessary for the proper splicing of exon 10 as well as the fact that protein SRp20 lowered the integration of exon 10 into wild-type and mutant tau pre-mRNAs (Yu et al, 2004). It was determined that a minimum of 1,294 base pairs of intron 10 is required for proper splicing of exon 10. The system was designed to identify compounds that can reverse *tau* alternative splicing (i.e. inclusion of exon 10) through biological molecules such as SRp20 protein. The system is shown in Figure 6.

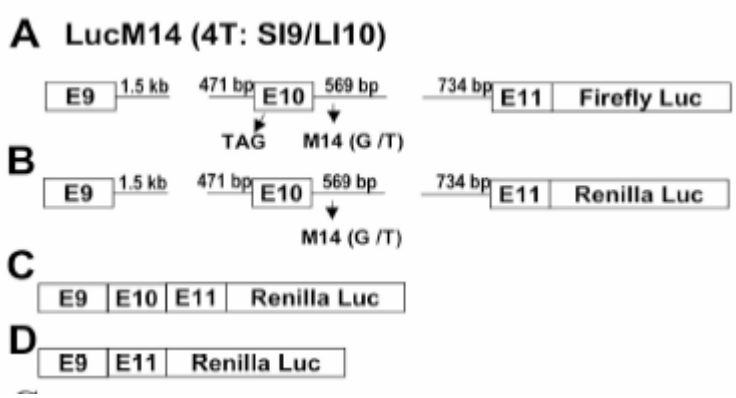

Figure 6: Design of the Cell-Based Luciferase System Used by Dr. Zhou's Lab to Evaluate Tau Exon 10 Splicing. Panel A shows a luciferase mini-gene construct with firefly luciferase (right side) fused downstream of Tau exon 11. The point mutation M14 (G to T) in intron 10 increases the inclusion of exon 10. Exon 10 inclusion into this construct would introduce a stop codon (TAG) by changing A to T, causing luciferase to not be expressed. Panel B shows the control mini-gene construct similar to Panel A but without the mutation in intron 10, so exon 10 inclusion will be minimal. Panels C &D show the structure of the constructs with and without exon 10, respectively (Yu et al., 2004).

The first plasmid construct in the screening system (Figure 6A) included a firefly luciferase reporter fused downstream of *tau* exon 11. Two mutations were made in the *tau* gene. The first base change was in exon 10, and generates a stop codon that does not alter exon 10 splicing patterns, while the second base change is a point mutation in intron

10 (labeled M14 in the figure) that produces an increase in exon 10 inclusion, to create a plasmid construct which would help recognize the molecules that encourage exclusion of exon 10. Without the point mutation it would be difficult to identify compounds using the wild-type form of the gene since most wild-type mRNAs from *tau* exclude exon 10. With this arrangement of the construct, when exon 10 is included (previously correlated with increased chance of a disease state), translation discontinues at the stop codon of exon 10, prohibiting the production of luciferase. When exon 10 is excluded from the mRNA (via an active therapeutic), translation continues through exon 11 reaching the luciferase gene. Therefore desirable drugs that induce an increase in the exclusion of exon 10 will generate an increased luciferase signal (Yu et al., 2004).

The second plasmid in the screening system (Figure 6B) is a control construct that used a comparable plasmid mini-gene to the one previously described, with the exception that the control construct does not contain the stop-codon, and the firefly luciferase is substituted with Renilla luciferase, which is minimally affected by the exclusion/inclusion of exon 10. This control mini-gene construct was cloned in a hygromycin expression vector.

Using the two constructs, stable cell lines containing the plasmids were made in neuroblastoma SKN-MC cells using neomycin and hygromycin as selectable markers. These stable transfected cell lines were used to demonstrate the minimum length of *tau* exon 10 required for correct splicing (Yu et al., 2004), and were also used by the Zhou lab in unpublished findings to screen 1,040 FDA approved drugs by luciferase levels and *tau* RT-PCR for exon 10 exclusion.

PROJECT PURPOSE

As stated in the Background, specific tauopathies have previously been associated with the inclusion of exon 10 in *tau* splicing. The purpose of this project was to use the plasmid based system of Yu et al, 2004, to further analyze five initial drugs that appeared to increase exon 10 exclusion in their original screen of 1,040 FDA approved compounds (unpublished data). Initially, the luciferase activity induced by certain compounds will be measured, then the potential positives will be validated by RT-PCR of *tau* mRNA. The identification of pharmaceuticals that modulate *tau* splicing can be prospective treatments for patients with tauopathies (Yu et al, 2004).

METHODOLOGY

Cell Culture

Standard sterile technique was used to grow SKN-MC cells containing the luciferase plasmid system described in Yu et al., 2004, for screening *tau* exon-10 exclusion. Persistent cell death proved to be a problem that occupied an extensive portion of the time spent in the lab. Therefore, the experimentation of different compositions of media was required to allow for cell viability. At first it was thought to be a contamination problem, but the cells continued to die after the incubator was decontaminated. First the percentage of penicillin/streptomycin antibiotic mixture was increased from 1% to 2%. Although some of the cells began to proliferate, they were not growing fast enough. Subsequently, the amount of fetal bovine serum was increased from 10% to 12.5%. The cells began to proliferate at a satisfactory rate necessary for experimentation.

The cells were finally maintained in a 10-cm Petri dish in Dulbecco's modified Eagle's medium supplemented with 12.5% fetal bovine serum and a 2% penicillin/streptomycin antibiotic mixture. These cells were allowed to grow to confluency and then were split to a new 10-cm Petri dish with a cell concentration of $1x10^6$ cells per mL.

Luciferase Assay

The cells were washed twice with PBS, then removed from the 10-cm Petri dishes using Trypsin. Using sterile technique, $2x10^4$ cells per 200 μL of medium were

added to 45 wells of a 96-well plate. The plates were covered and placed in a 37°C, 5% CO_2 humidified chamber overnight to allow for cell adhesion. Five FDA approved drugs (Microsource Discovery System, Inc.) previously identified by Zhou's lab as decreasing exon-10 inclusion (Colchicine, Podophyllotoxin, Gentian Violet, Peruvoside, and Sanguinarine Nitrate) were dissolved in DMSO to achieve a concentration of 1 M. These FDA approved drugs were added to the cells in 1, 5, and 10 μM concentrations. A 1% DMSO control was also used.

The cells were then allowed to incubate at 37°C for 24 and 48 hours. After these time periods, the cells were washed with PBS, which was then removed. A commercial source luciferase lysis buffer was then added to the cells. The plates were then incubated to allow for cell lysis. The 96-well plate was then placed in a Perkin Elmer EnVision 2102 multilabel reader, and light emission monitored at 700 nm. This experiment was repeated twice.

RNA Extraction

The cells were washed twice with PBS, and removed from the 10-cm Petri dishes using Trypsin. Using sterile techniques, $1x10^6$ cells were added per 1mL medium to each of the 6 wells. The cells were allowed to adhere and grow overnight in a 37°C, 5% CO_2 humidified chamber.

Cells were again washed twice with PBS, then lysed directly in the culture by adding 0.5mL Trizol (Invitrogen, Carlsbad, CA, USA). The cell lysate was passed several times through a pipette, to thoroughly lyse the cells and extract the RNA into the aqueous phase. The samples were incubated for 5 minutes at 15-30°C to allow complete

dissociation of nucleoprotein complexes. The contents of each well were transferred to a separate 1.5mL eppendorf tube. Next, 100 µL of chloroform was added to each tube, and the tubes were shaken vigorously by hand for 15 seconds. The samples were then incubated at 15-30°C for 2-3 minutes. The samples were then centrifuged at 12,000 x g for 15 minutes at 2-8°C to separate the aqueous and organic liquid phases. The upper aqueous phase was then removed to a fresh tube and mixed with 0.25 mL isopropyl alcohol. The samples were then allowed to stand at 15-30°C for 10 minutes followed by centrifugation at 12,000 x g for 10 minutes (2-8°C) to pellet the total cellular RNA. The supernatant was discarded and the RNA pellet was washed with 0.5 mL of 75% ethanol. Each sample was mixed by vortexing, then centrifuged at 1500 x g for 5 minutes (2-8°C). The RNA pellet was allowed to air dry for approximately 5-10 minutes, and if not completely dry was dissolved in RNase-free water. The samples were analyzed by a spectrophotometer at optical densities of 260nm and 280nm. The ratio of OD260nm/OD280nm had to be greater than 1.6 for the RNA to be pure enough to proceed. The samples were then stored at -80˚C until reverse transcription.

This experiment proved to be difficult due to the continued cell death. Therefore, the quality of RNA was diminished to the point where it was unusable. Four attempts were needed to achieve the proper optical density ratio necessary to proceed with reverse transcription.

Reverse Transcription

One microgram of extracted RNA from each well was added to 1µL of oligo(dT) primers, with a forward sequence of 5'GCGAATTCGGTGAACCTCCAAAATCAGG-

GGATCG-3' and a reverse Luciferase 200 primer with the sequence 5'-ATAGTCTCT-GCCAACCGAAC-3.' RNase-free water was added until a final volume of 5 μL was achieved. The tubes were then placed in thermocycler at 70°C for 5 minutes to denature the RNA, followed by incubation at 4°C for 5 minutes, and spin cycle for 10 seconds (to pellet the solution in the tube). Each 5 μL mixture was added to 4.5 μL RNase-free water, 4 μL buffer, 4 μL $MgCl_2$, 1μL dNTP, 0.5 μL RNase inhibitor, as well as 1μL reverse transcriptase (Promega). Each tube was placed in the thermocylcer for 1 cycle consisting of 5 minutes at 25°C, 1 hour at 42°C, and 15 minutes at 70°C (an increasing temperature series of reverse transcription). The cDNA samples were then stored at -20°C until PCR.

PCR

For the polymerase chain reaction, 12.5 μL of Choice Blue Master Mix(Denville Scientific Inc.) containing dNTPs and taq polymerase were added to 0.5 μL of template, 10 μL of water, 1 μL of forward primer, and 1 μL of reverse primer. The sample was then heated at 94°C for 5 minutes. Next, 30 cycles of the following temperatures and times were conducted: 94°C for 45 seconds, 50°C for 45 seconds, and 72°C for 1 minute 30 seconds. Next, a 10 minute 72°C polishing step followed. Following this reaction, 10 μL of the reaction mixture were electrophoresed on a 1.5% agarose gel for 60 minutes.

RESULTS

The main objective of this project was to confirm the previous unpublished identification by Zhou's lab, of five FDA approved pharmaceuticals that minimize the inclusion of *tau* exon 10. This was accomplished by using a cell based luciferase assay system developed by Yu et al., 2004, and was confirmed by the use of *tau* RT-PCR.

Luciferase Assay

Initially, conditions had to be developed for growing the transfected cells. Persistent cell death proved to be a problem that occupied an extensive portion of the time spent in the lab. This was thought to be a contamination problem, but the cells continued to die after the incubator was decontaminated, so the percentage of penicillin/streptomycin antibiotic mixture was increased from 1% to 2%. This did not solve the problem, so the fetal bovine serum was increased from 10% to 12.5%, which finally allowed the cells to grow at a satisfactory rate necessary for experimentation.

Of the five compounds tested: Colchicine (11-8C), Podophyllotoxin (2-7H), Gentian Violet (3-7A), Peruvoside (7-4H), and Sanguinarine Nitrate (5-9E), all compounds proved to statistically increase luciferase activity. A sample of the results of the luciferase assay is shown in Table I, and these results are represented in a histoplot in Figure 7.

Table 1: Sample Luciferase Assay Data for Five Compounds Tested for 24 Hrs

10 µM			5 µM			1 µM			Compound
4532	5336	5444	7780	5584	6040	1936	1160	1160	**3-7A**
1872	776	1180	4000	4520	4696	1160	1212	1196	**5-9E**
2340	2708	2816	3252	2956	2832	1644	2144	2468	**11-8C**
5236	5076	5200	3984	3816	3516	2472	2600	2984	**7-4H**
2132	2612	3436	2184	2800	2508	2676	2288	1400	**2-7H**
1240	1496	1836	1476	1588	1612	984	1196	1068	**Control**

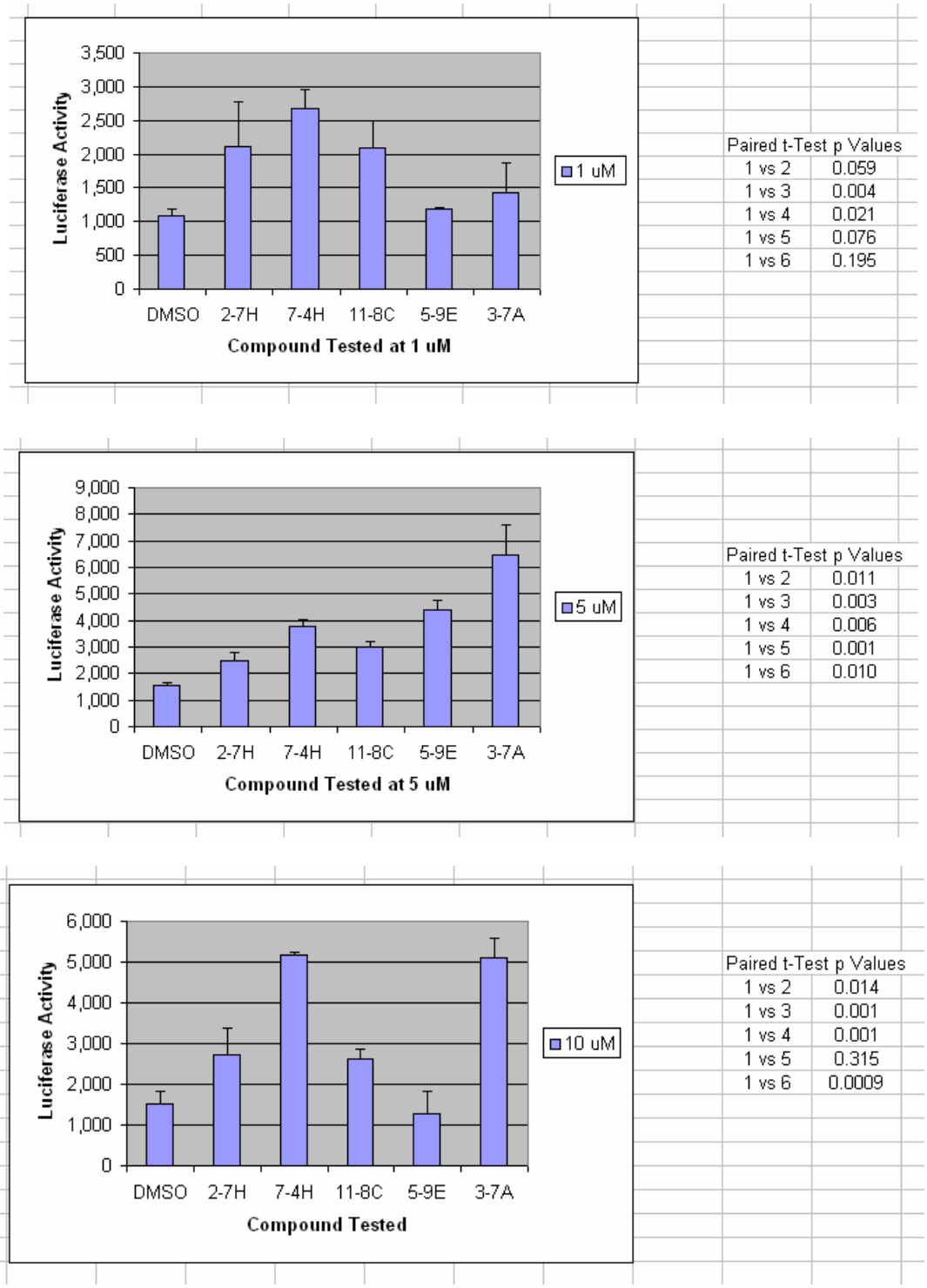

Figure-7: Histoplots of Luciferase Activity Versus Drug Concentration for the Five Compounds Tested. Upper panel denotes 1 uM compound concentrations; middle panel, 5 uM; and lower panel 10 uM. Each histobar denotes the means of 3 independent trials. Error bars denote one standard deviation. P values were calculated using a pairwise t-test.

As seen in Figure-7, at 1 µM concentration (upper panel) all compounds tested showed average luciferase activities above the DMSO control, however only 7-4H and 11-8C showed statistically signficant elevations ($p < 0.05$) using a pairwise t-test. Compound 7-4H was the most active at this concentration. At 5 µM concentration (middle panel) all five drugs showed statistically significant increases ($p < 0.05$) above the DMSO control. At this concentration, all compounds except 2-7H showed higher levels of luciferase activity than at 1 µM. Compound 3-7A (GentianViolet) was the most active at this concentration. At 10 uM concentration (lower panel) all compounds tested except 5-9E showed statistically significant increases above the DMSO control, with 3-7A and 7-4H being the most active at this concentration. However only compound 7-4H showed higher luciferase activity than at the 5 µM level. The decreased luciferase activities for the other compounds could be due to a general cell cytotoxicity at high concentrations.

RT-PCR

RT-PCR was performed to verify that the alternative splicing of *tau* exon 10 actually occurred in the cells that displayed the increased luciferase activity in Table 1. The extraction of total cellular RNA proved to be a difficult task from the cell cultures showing high levels of cell death. The quality of RNA was diminished to the point where it was unusable. Four attempts were performed with those samples, none of which worked. Eventually the medium problem was solved, producing viable cells and good RNA, but the gel conditions used did not fully resolve the two alternative splicing amplicons (included and excluded exon-10) resulting in improper band separation. So a

previous RT-PCR result obtained by the Zhou lab (Figure 8) was used which shows a predominant lower band (denoting exon-10 exclusion) for cells treated separately with each of the five compounds (lanes 1-5). More of the mRNA from the cells treated with these five compounds excluded exon 10 rather than included it, as seen in the more intense lower band labeled –E10 in Figure 8. RT-PCR from control cells treated with DMSO vehicle (lane 6) shows a slightly upper band intensity denoting the inclusion of exon 10.

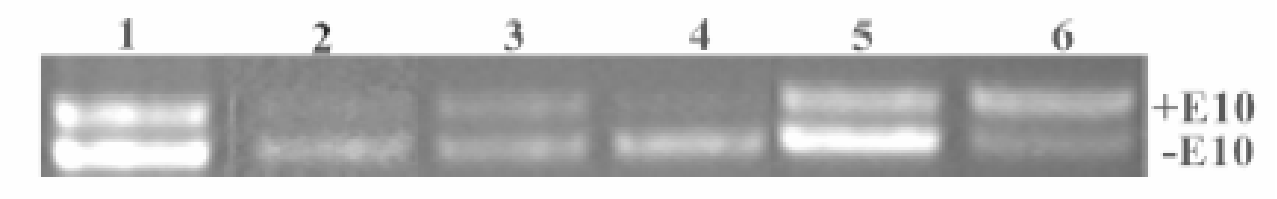

Figure 8: RT-PCR of *Tau* Exon 10 Region. mRNA from cells treated separately with each of the five compounds was subjected to RT-PCR for *tau* exon-10, then electrophoresed. Lanes are as follows: Colchicine, Podophyllotoxin, Gentian Violet, Peruvoside, Sanguinarine Nitrate, and DMSO control respectively. Note the stronger band in the lower position corresponding to mRNA without exon-10 in lanes 1-5, with the exception of the DMSO control (lane 6).

DISCUSSION

The main conclusion of this project was that all five compounds tested were able to statistically significantly increase luciferase levels above DMSO vehicle control at 5 µM concentrations, validating the initial unpublished compound screen done by Zhou's lab. The highest levels of luciferase activity were obtained for compounds 7-4H and 3-7A at the maximal concentrations tested here. The increased luciferase activities for all five samples were accompanied by a preponderance of tau mRNA without exon-10 as verfied by RT-PCR. This data shows that such compounds can increase the exclusion of *tau* exon 10, which presumably would lead to a decrease in the production of the larger more toxic tau protein in neuronal cells.

As explained in methodology, time courses used during the luciferase assay experiment included 24 and 48 hours. After 48 hours no luciferase activity was observed (data not shown). This could be due to the fact that the time period was too long and toxic to the cells, especially in the presence of DMSO vehicle, resulting in death. Similarly, it was observed that at high concentrations of the compounds the cells died, suggesting that the concentration was too toxic for the cells. It may have been more beneficial to try a lower concentration for a longer period of time because a lower concentration of a toxic drug will be necessary for human trials.

An RT-PCR experiment previously conducted in the Zhou lab was shown in this MQP due to the author's failed RT-PCR attempt. The author's RT-PCR attempt was not electrophoresed long enough, and no band separation occured. In addition, the exposure time of this gel was also too long, resulting in extremely dark, unresolved bands.

However, the RT-PCR result used in this MQP showed that the five samples that showed an increase in luciferase activity also showed greater amounts of tau exon-10 exclusion, further validating the luciferase assay developed by Yu et al., 2004.

Compound 3-7A (Gentian Violet) is known to be a toxic chemical, it is important to test other chemicals with similar structures to see if they could be as effective but with less cellular toxicity. Currently, Jianhua Zhou's lab at the University of Massachusetts Medical School is performing High Throughput Screening (HTS) of about 20,000 chemicals to see if more hits can be discovered. The hope of HTS is that a certain family of drugs will be found to minimize the exclusion of exon 10, which would increase our understanding of the *tau* splicing mechanism, and lead to the design of less toxic pharmaceuticals for treatment of tauopathies.

In summary, in this project all five compounds tested proved to significantly increase the exclusion of tau exon 10 at the 5 µM concentration level. This MQP achieved its overall goal of validating the compounds originally identified by Zhou's lab as facilitating the exclusion of tau exon-10. In the future, similar compounds may be beneficial in the treatment of tauopathies.

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