Inhibition of I1PP2A as a Therapeutic Strategy for Tauopathy

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Tauopathies—a class of neurodegenerative diseases, the most notable of which is Alzheimer's disease (AD)—are characterized by the hyperphosphorylation of tau, a protein responsible for stabilizing microtubules inside cells (primarily in the CNS). I1PP2A, an endogenous protein inhibitor of the most prominent tau phosphatase, PP2A, is known to be elevated in AD. Heightened levels of I1PP2A alone could account for the hyperphosphorylation of tau, making it a prime target for treatment. The goal of this project was to develop an inhibitor of I1PP2A that has potential as a therapeutic treatment and show that treatment with this inhibitor will increase PP2A activity levels. Increasing I1PP2A concentration in mouse hippocampal cell lysates (to simulate tauopathy) decreased the activity of PP2A by an average of 19% ($p = 0.073$), as expected. Two possible inhibitors of I1PP2A—its antibody and a batch of RNA aptamers selective to I1PP2A (developed with SELEX and his-tagged I1PP2A bound to Ni-NTA columns)—increased PP2A activity by an average of 22% when treated to these lysates ($p = 0.209$). These observed trends lend support to our hypothesis. The RNA aptamers generated have therapeutic potential as they may be able to be expressed inside neurons affected by tauopathy if DNA complements were successfully packaged into rAAV vectors and transfected into the cells.

INTRODUCTION

Tauopathies are a class of devastatingly progressive neurodegenerative diseases—the most notable and prevalent is Alzheimer's disease (AD)—for which no effective treatment currently exists. The shared feature of this group of conditions is the accumulation of polymers formed by the protein, tau [1]. In Alzheimer's disease specifically, two features are diagnostic: one is the formation of plaques formed by amyloid precursor protein (APP) and other cellular debris, and the other is the formation of paired helical filaments (PHF) of tau contained in neurofibrillary tangles. The plaques of APP are the subject of many research projects working toward treating tauopathy; however, they are not present in all tauopathies, and the presence of abnormal tau is more closely correlated to memory difficulties and dementia (prime symptoms of tauopathy) [2].

Tau's function is to stabilize microtubules through binding to tubulin, though tau can exist in various forms of phosphorylation, which alters its performance. Hyperphosphorylation causes tau to dissociate from tubulin, causing microtubule destabilization and PHF formation (Figure 1). Bundles of destabilized microtubules (neurofibrillary tangles) interfere with normal neuronal cell functions, such as synaptic transmission and intracellular transport, and can lead to cell death [3] (Figure 2).

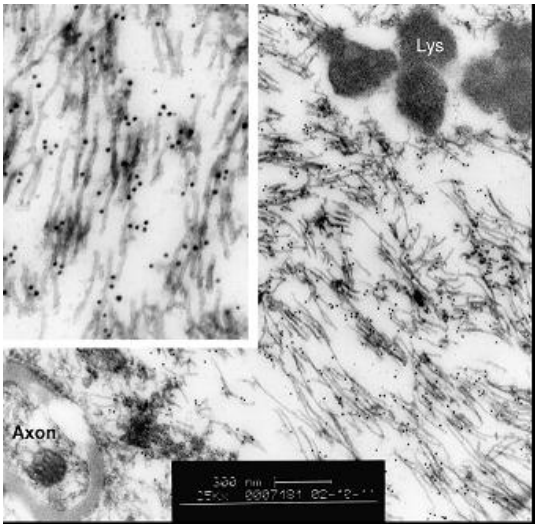

Figure 1. Microscopic view of abnormal bundles of tau.

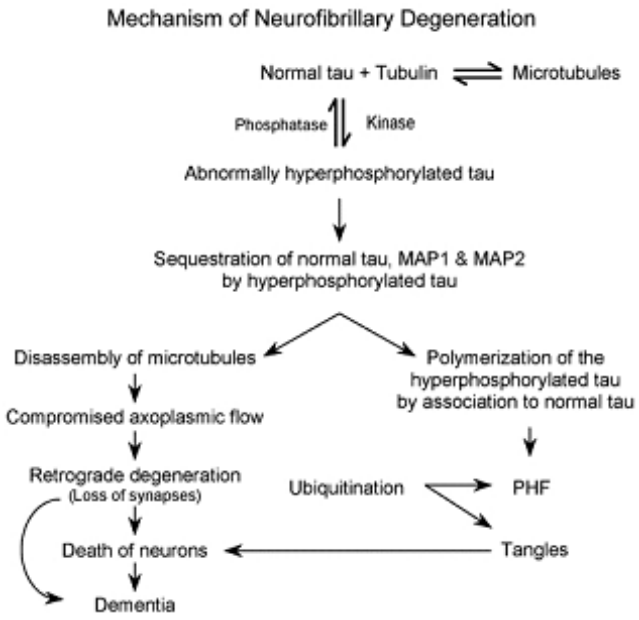

Figure 2. Mechanism of typical development of tauopathy.

Tau is known to contain many phosphorylation sites [4], though several serine (ser) and threonine (thr) residues are known to be directly involved with normal tau function. Increased phosphorylation on these sites alone has shown to alter the function of tau and cause increased PHF formation [5]. Many phosphatases exist in neuronal cells, which act specifically on ser/thr residues, though the most prominent is protein phosphatase 2A (PP2A) [6]. Dephosphorylating tau is not nearly PP2A's only function, as it is also responsible for regulating signaling pathways in concurrence with other kinases and phosphatases, and even for stabilizing microtubules directly [7], [8]. Decreased activity of PP2A, which is observed in AD [9], has been hypothesized to account for tau hyperphosphorylation [10], though the reason for this diminished activity is not certain.

An endogenous inhibitor of PP2A, named I1PP2A (also called ANP32A or PHAP1), has been shown to be elevated in AD [11]. The increased inhibition of PP2A by I1PP2A alone could hypothetically account for diminished PP2A and tau hyperphosphorylation, though I1PP2A has also been observed to compete with tau for microtubule binding sites [12], increasing its likelihood as being a major component in tauopathies. Based on these findings, I1PP2A looks to be an attractive target for treating tauopathy, though no suitable drug candidates are known to exist. Computational drug design could be utilized to develop a therapeutic inhibitor to I1PP2A, though this method is very expensive and laborious. The use of aptamers, however, has shown to be an economical, efficient, and clinically-safe alternative [13].

Aptamers are small oligonucleotides (typically 20-100 nucleotides in length) that are selected from a large random sequence pool for binding to a target with high affinity and specificity [14]. DNA strands synthesized through phosphoramidite chemistry can allow nucleotides to be incorporated into a sequence at random. With this strategy, 10^{13} different combinations of sequences can easily be generated for oligonucleotides only 30 bases in length. The recursive SELEX (Systematic Evolution of Ligands by Exponential Enrichment) process is employed to select aptamers out of such a library on the basis of affinity to a target molecule. Each round of SELEX develops a batch of aptamers more selective to the target than the previous, and often 10 rounds of selection are sufficient to isolate the best candidate aptamers [15]. This method has been altered to incorporate many different selection techniques, such as capillary electrophoresis [16]; surface plasmon resonance [17]; and, similar to what is used in this project, Ni-NTA beads [18].

Numerous difficulties arise when attempting to deliver a drug to specific areas in the body, especially the brain. An effective therapeutic agent must successfully be able to cross the blood-brain barrier and pass through cell membranes intact if it is delivered through the bloodstream. The use of recombinant adeno-associated viral vectors (rAAV) may be a good solution to some of these problems and can be developed from just about any oligonucleotide of appropriate size [19], such as the aptamer selected for I1PP2A. AAV is capable of transfecting non-dividing cells, such as mature neuronal cells implicated in tauopathies), and have even been used recently in clinical trials for other neurological diseases

[20]. Although treatment with AAV currently requires invasive surgical procedures, the permanent nature of AAV transfection still makes it an attractive treatment strategy (regardless of the fact that no other treatment exists).

HYPOTHESIS

Increased levels of I1PP2A may be the cause of at least some forms of tauopathy through the inhibition of PP2A and subsequent hyperphosphorylation of tau. If other pathologies are causing diminished PP2A activity or there are other reasons for tau hyperphosphorylation, decreasing I1PP2A levels could still compensate for this imbalance. The inhibition of I1PP2A should restore PP2A activity to normal levels in tauopathies, thus serving as an effective treatment strategy.

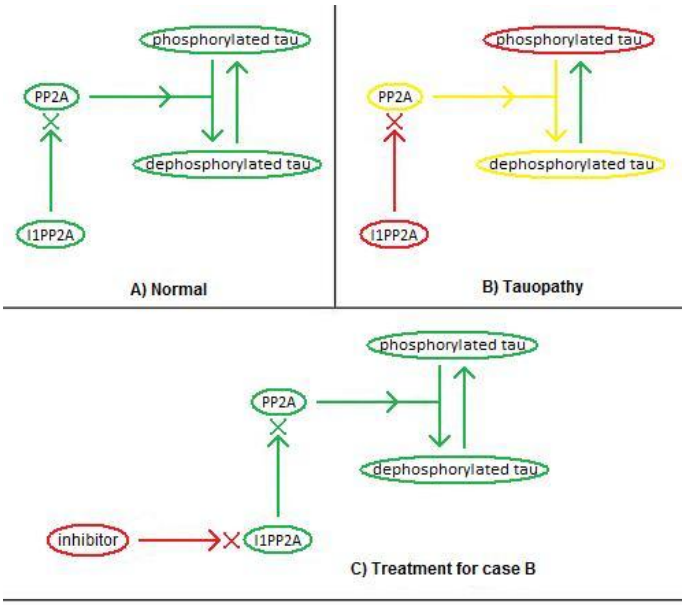

Figure 3. Possible conditions of tau regulation. Green indicates normal levels, whereas red and yellow indicate high and low levels, respectively. Case A: the properties of normal tau phosphorylation. Case B: tauopathy due to an increased amount of I1PP2A, which subsequently causes increased inhibition of PP2A and decreased tau dephosphorylation. Case C: the addition of an I1PP2A inhibitor in case B decreases its levels to normal and balances out all other conditions.

EXPERIMENTAL METHODS

I1PP2A Production and Purification

For this project, many assays and procedures required the use of I1PP2A, which is not economically available in large quantities. This required production of I1PP2A in our own lab. Plasmids incorporating a gene for recombinant, histidine-tagged I1PP2A production were ordered for this purpose (QAIgenes E. Coli Expression Kit – ANP32A). The protocol included with the kit was followed for transfection of competent E. coli cells, growth of these cells, and induced expression with IPTG. Following expression, cell cultures were lysed with lysozyme and Benzonase nuclease and centrifuged to pellet the cellular debris, as directed by the kit. I1PP2A in the supernatant was purified using Ni-NTA spin columns (QAIGEN 3014), following this kit's protocol and using the supplied reagents. Samples of each step in the purification (3 washes, 4 elutions) were saved for analysis by gel electrophoresis.

To remove the I1PP2A from the high salt imidazole buffer, each eluate was passed through columns filled with Sephadex G-25 and washed with RNase-free, DEPC-treated water. 100 µL sections of eluate from these columns were saved for determination of protein concentration.

SDS-PAGE and Western Blots

10 µL of samples to be analyzed by electrophoresis were mixed with 9.5 µL of Laemmli buffer and 0.5 µL of 2-mercaptoethanol and placed in a boiling water bath for 5 minutes (to denature the protein). The samples were then pipetted into a precast 4-15% Tris HCl Ready Gel (Bio Rad 161-1176), which was loaded for electrophoresis set at a constant 200 V. Sample and running buffers were used as directed in the Bio Rad Application Guide. The gel was removed from the machine after the dye front reached the end of the gel.

Gel staining was accomplished using Coomassie Brilliant Blue G-250 Stain Solution (Bio Rad). The protein was fixed to the gel by soaking in a 50% methanol, 10% acetic acid solution for 1 hour. The gel was the transferred to the G-250 staining solution for 4 hours in an orbital shaker. Afterwards, the gel was destained with a 5% ethanol and 7.5% acetic acid solution overnight.

For western blot analysis, the protein was transferred to a PVDF membrane (pre-soaked in methanol) by stacking it on gel and placing in an electrophoresis transfer block, set to 80 V for 4 hours. The PVDF membrane was then removed from the block and washed with transfer buffer (0.1 %v/v Tween 20 in PBS). Non-specific binding sites were blocked by placing the membrane in transfer buffer with 5% nonfat milk for 1 hour in an orbital shaker. After 2 rinses with the transfer buffer, the primary antibody (Rabbit Anti-PHAP1, Chemicon International AB4515), diluted in transfer buffer, was added to the membrane and incubated again for 1 hour. This was followed with 3 more washes with transfer buffer (for 10 minutes each), after which a secondary antibody (Anti-mouse HRP) was added to the membrane and incubated for 1 hour. Three additional washes were done, followed by rinsing with dH_20. Reagents from an ECL Detection kit (GE Healthcare RPN2132) were added to the membrane as stated in the included protocol and incubated for 5 minutes. Chemifluorescence was detected with a storm molecular imager.

Protein Concentration Assay

All protein concentrations were determined using a Bio-Rad DC Protein Assay kit (a variation of the Lowry assay). A protein standard curve was prepared by diluting albumin to 5 known concentrations (0.2, 0.525, 0.85, 1.175, and 1.5 mg/mL). 5 µL of each sample to be analyzed was loaded into a microtiter plate. Reagents included in the kit were added as directed in the kit's protocol. After mixing and a 30 minute wait period, absorbances were read in a standard microplate reader at 750 nm. Absorbances were compared to the standard curve to determine sample protein concentrations.

PCR, RT-PCR, and Transcription

Polymerase Chain Reaction (PCR) was carried out using a Phusion High-Fidelity PCR kit (New England Biolabs F-5535). Reaction mixtures (50 µL) were created using the reagents included with the kit and following the kit's protocol. The reaction mixture was subjected to 30 rounds in a thermal cycler at temperatures of 95˚C, 55˚C, and 72˚C for denaturation, annealing, and extension, respectively. To purify dsDNA from the reaction mixture, QIAquick PCR Purification kits (QIAGEN 28104) were used. All reagents and materials needed were included in the kit, and its standard protocol was followed.

Reverse Transcription PCR (RT-PCR) was accomplished with USB One-Step RT-PCR Kits (Affymetrix 78350). A 50 µL reaction mixture was created following the protocol and using the reagents included in the kit (enzyme mix, nucleotide mix, buffer and RNase inhibitor), starting with up to 6 µL of an RNA sample. The mixture was then placed in a thermal cycler for 40 rounds, under the same conditions used for PCR. QIAquick PCR purification columns were also used to purify dsDNA from these reactions.

To transcribe DNA oligonucleotides to RNA, Ambion MEGAshortscipt High-Yield Transcription kits were used (Applied Biosystems AM1354). Reaction mixtures were created following the kit's protocol and using supplied reagents, then incubated for 4 hours at 37^{o}C. Alcohol precipitation was used to recover the RNA from this solution, also following the kits protocol.

SELEX and Aptamer Selection

To begin the aptamer selection process, a library of DNA aptamers was ordered with known sequences, as shown in Figure 4. Forward and reverse primers were also needed for these aptamers.

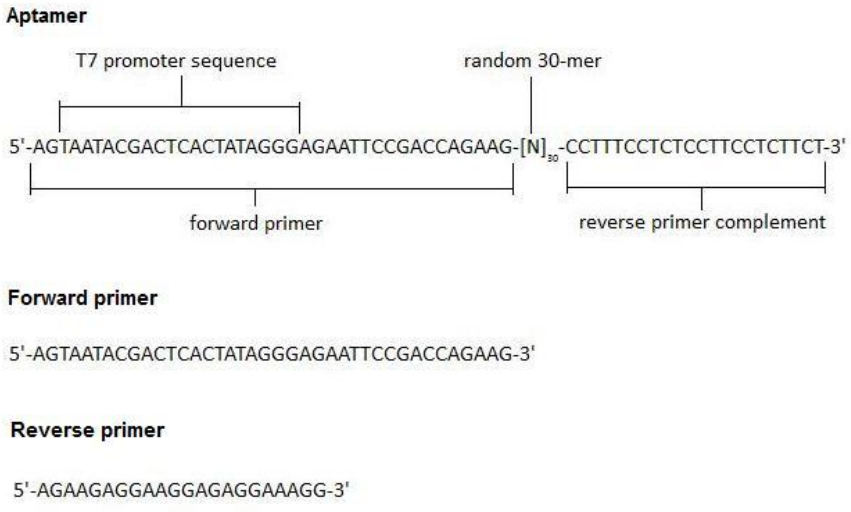

Figure 4. DNA aptamer sequence contains 30 random nucleotides (marked by the letter N), flanked by forward and (complement) reverse primer sequences. A T7 promoter sequence is included in 1st half of the forward primer, making transcription of the aptamers to RNA simple. Forward and reverse primers sequences are also shown.

The DNA aptamer library was first transcribed into RNA. The batch was then incubated with 1 mL of a 50% slurry of Ni-NTA agarose in 30% ethanol for 30 minutes at 4^{o}C with mild shaking. This slurry was then loaded into a 1 mL polyethylene column (with a frit to prevent the agarose from passing through column) and washed with water to recover the flow-through. This batch of aptamers had thus been screened to remove any aptamers that bind directly to the Ni-NTA. After 2 of these screenings, the batch of RNA aptamers was incubated with Ni-NTA and a known amount of I1PP2A and loaded into a 1 mL column. The histidine-tag on the I1PP2A binds to the Ni-NTA, preventing it from

washing off the column, and also keeps any bound aptamers held to the column. The column was then washed with water to remove any aptamers not bound to I1PP2A. The I1PP2A on the column was then eluted with 250 mM imidazole, which competes with the his-tag for binding to nickel, removing any bound aptamers from the column as well. A sample of the eluate was sent through RT-PCR and purified for dsDNA, which generates a batch of DNA whose RNA complement is more selective to I1PP2A than the previous batch. This process was repeated 10 times to isolate aptamers with very high affinity to I1PP2A, as shown in Figure 5.

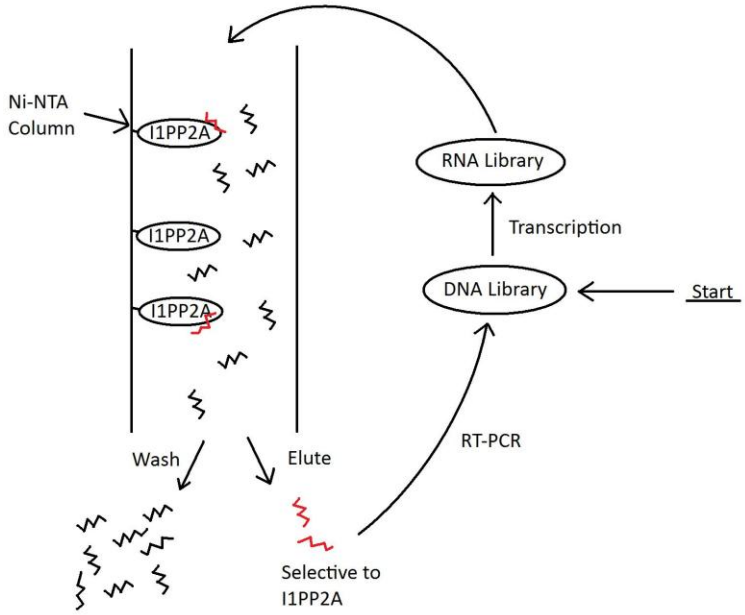

Figure 5. Process of aptamer selection used in this project. In the first 4 rounds of this process, 10 µg of I1PP2A was incubated with Ni-NTA and the batch of aptamers. To increase the stringency of aptamer selection, this number was reduced to 6 µg in rounds 5 – 7 and 2 µg in rounds 8–10.

PP2A Activity Assay

PP2A activity was measured using a RediPlate™ 96 EnzChek® Serine/Threonine Phosphatase Assay Kit (Molecular Probes R-33700). To prepare a fluorescent standard curve, 100 µL of reaction buffer (included with the kit) was added to each well of the reference standard strip of the microplate, then mixed to solubilize the substrate in the wells. Samples to be analyzed were diluted to 100 µL with reaction buffer and added to the microplate (also with mixing). Negative controls were created by adding only reaction buffer to blank wells.

The microplate was then incubated at room temperature (guarded from light) for 30 minutes, after which fluorescence was read with a fluorescence microplate reader, set to excitation and emission wavelengths of 355 and 460 nm, respectively. The fluorescence related directly to the amount of PP2A activity in the sample.

RESULTS

The SDS-PAGE gel, shown in Figure 6, shows bands at approximately 28 and 56 kD. I1PP2A is known to be 28 kD in size, so these bands could be monomers and dimers of I1PP2A, respectively. No bands are visible in wash 3, showing that only 2 washes were necessary. Four elutions were completed in total, though the 3rd and 4th had no visible bands and are not shown. Nearly all of the I1PP2A appeared to wash off the column in the 2nd elution.

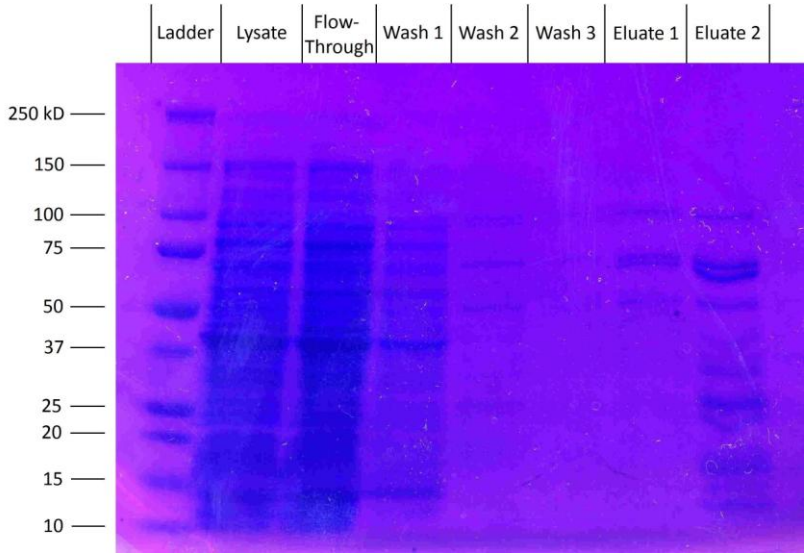

Figure 6. SDS-PAGE gel of the purification of I1PP2A from E. coli lysates. Lane 1: protein ladder of known sizes. Lane 2: cell lysates. Lane 3 flow-through of the lysate passed through the Ni-NTA column. Lane 4: sample of 1st wash with 10 mM imidazole. Lanes 5 and 6: 2nd and 3rd washes with 20 mM imidazole. Lanes 7 and 8: 1st and 2nd eluate with 250 mM imidazole.

A western blot of the 2nd elution shown in Figure 7 confirms the identity of I1PP2A. Lane 2 only shows 1 significant dark band at approximately 56 kD, likely indicating an I1PP2A dimer. Lanes 3 and 4 have darkest bands at 28 kD and 56 kD, as expected. Many other lighter bands showed immunoreactivity with the primary antibody (specific for I1PP2A), indicating that the majority of the bands in the 2nd elution of Figure 6 are indeed fragments or complexes of I1PP2A, signifying high purity of the desired protein.

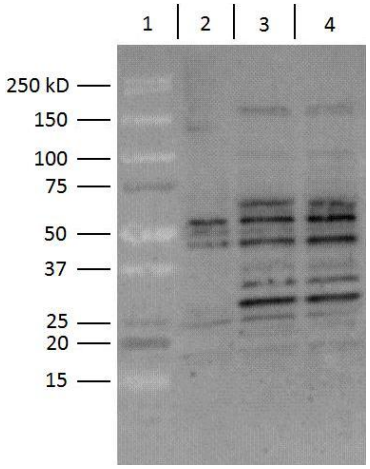

Figure 7. The western blot of a sample from the 2nd elution of purified I1PP2A (shown in Figure 6), run on an SDS-PAGE gel. Lane 1: the standard protein ladder. Lane 2: a sample of I1PP2A, which was not boiled and no 2-mercaptoethanol was added prior to being run on the electrophoresis gel. Lane 3: a sample with mercaptoethanol added but was not boiled. Lane 4: a sample with mercaptoethanol added and boiled for 5 minutes.

The results of the PP2A dephosphorylative activity assay are shown in Figure 8. The addition of extra I1PP2A significantly decreased the activity of PP2A in all cases except for the 1st set. The addition of PHAP1 antibody increased PP2A activity, and the addition of the final batch of aptamers increased it even further.

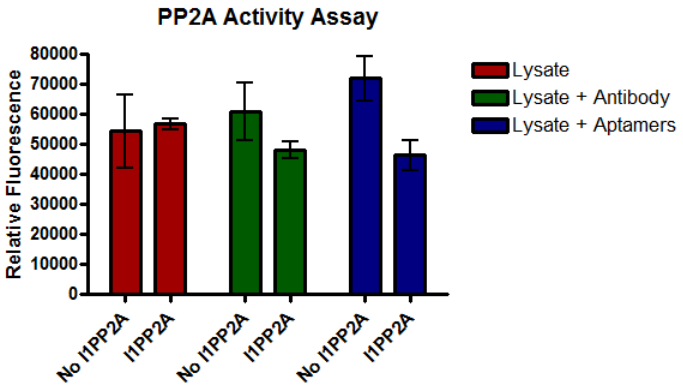

Figure 8. Displays the amount of fluorescence detected for each sample labeled. Each column shows the average and standard deviation of 3 duplicate measurements. The red columns contained only cell lysates, incubated with or without 1 µg of added I1PP2A. The green columns show lysates incubated with 1ug of an I1PP2A antibody, with or without I1PP2A, and the blue columns show lysates incubated with the aptamer batch (amount not known) after the 10th round of SELEX, with or without added I1PP2A.

DISCUSSION AND CONCLUSIONS

As shown in Figures 6 and 7, I1PP2A was successfully produced by transfection of E. coli cells, retained its

activity, and was recovered with good purity. Figure 7 shows that I1PP2A unfolds easily with 2-mercaptoethanol, without any need for boiling. An interesting observation is that without 2-mercaptoethanol it seems to exist almost exclusively in the dimer form. I1PP2A contains only 2 thiol groups in its entire structure, leading to the possible conclusion that a disulfide bond exists between molecules. Since no larger complexes seem to forms, it appears that I1PP2A contains only one of these thiol groups exposed to the environment, free to react with other thiol groups, while the other is well hidden inside the folded protein.

As shown in Figure 8, PP2A activity was decreased 19% on average upon the addition of extra I1PP2A ($p = 0.073$). The activity of PP2A somehow increased after the addition of I1PP2A in the plain cell lysate sample (2^{nd} red column). This may have been caused by monomeric I1PP2A becoming inactivated by dimerization with large amounts of added I1PP2A. Another reason could have simply been human error, though the experiment should be repeated in either case. The activity also appeared to increase in the presence of an antibody to I1PP2A, and more so by the addition of aptamers selected for I1PP2A ($p = 0.209$). The visible trend does lend support to our hypothesis, and it is encouraging that the aptamers may inhibit I1PP2A more successfully than the antibody. However, the amount of aptamers added to the samples were not known, so the large increase in activity in the last set of columns could have been due to the addition of a very large amount of moderately effective aptamers.

It appears that neither the antibody nor the aptamers were at all successful in counteracting the addition of extra I1PP2A. Since this is contradictory to the other results, too much I1PP2A may have been added to the sample to give informative results and may have simply drowned out the effects of the inhibitors. This experiment should also be repeated with different concentrations of added I1PP2A to determine if this is the case.

FUTURE WORK

From this batch of aptamers, which has shown to be selective to I1PP2A, individual aptamers will be selected and sequenced. These particular sequences will be tested individually using the PP2A activity assay performed earlier to determine which single aptamer works best and under what ranges of concentrations it does so.

If successful in screening a single aptamer that binds to I1PP2A with high specificity and affinity and shows promising pharmacological attributes, we will incorporate this oligonucleotide into a rAAV gene transfer vector for surgical delivery into the brain of adult rats. PP2A activity assays from these brain tissue extracts will determine the effect these aptamers have on enzymes *in vivo*.

If the aptamer vectors are successful in increasing PP2A activity, they will be surgically delivered to the brains of rodent Alzheimer's disease models. PP2A activity and disease pathology will be monitored, and spatial task acquisition and retention tests will be done on the transfected rats. This will test the final hypothesis that inhibiting I1PP2A and increasing dephosphorylation by PP2A will ameliorate the symptoms of tauopathy.

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