**Inhibition of prion-like properties in alpha-synuclein using promazine derivatives**

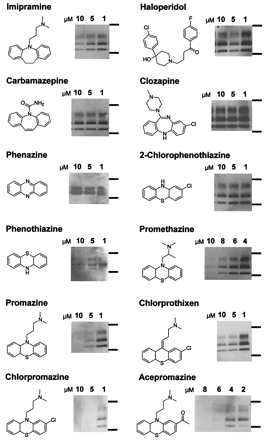
**Shwetha Kochi**

1. **Introduction**

Prions are protein particles composed of irregularly folded proteins that infect regularly folded proteins, causing them to misfold. They cause this infection by misfolding into beta sheet rich conformations that act as a template to promote conformational changes in regularly folded or wild type protein. This infection causes a chain reaction that further leads to neurodegeneration. Moreover, these abnormal proteins can be passed on to unaffected nerve cells, extending the neurodegenerative process.1

The fact that misfolded proteins play a role in the mechanism of Parkinson’s Disease has been proposed and experimented for a while, as this commonly known disease is characterized by the accumulation of alpha-synuclein aggregates in the axons of dopaminergic nerve cells in the substantia nigra and other parts of the central and peripheral nervous systems.2 Olanow & Brundin (2013) concluded, after analyzing a number of alpha-synuclein’s “prion hypothesis” studies, that alpha-synuclein’s fibrils/aggregates are possibly taken up by the neuronal axons, transmitted from neuron to neuron, and promote misfolding of host alpha-synuclein, causing neuronal dysfunction and formation of Lewy bodies.1 These findings are consistent with multiple other studies that conclude alpha-synuclein is a prion-like protein and Parkinson’s Disease is a prion-like disorder.1,2,3

The most well-known prion protein is PrPSc, the misfolded form of PrPC. Comparison studies between this prion protein and alpha-synuclein showed that both natively exist in the alpha-helix rich conformational state, but, when present in high concentration or near mutant forms, misfold to form beta-sheet rich conformational proteins. Both proteins are prone to aggregation in their mutant forms, whereas their native states are resistant to aggregation.4 No inhibitors have been found that prevent or resist this misfolding cascade of alpha-synuclein that results in the creation of Lewy bodies and neurodegeneration. However, multiple studies have been performed on PrPSc prions that have found potent inhibitors. Because this proposal is focused on studying prion effects that cause neurodegeneration, one main study that focuses on inhibitors that can cross the blood brain barrier will be discussed.

Korth, May, et al. (2001) report that tricyclic derivatives of acridine and phenothiazine show half-maximal inhibition of PrPSc formation in cultured cells infected chronically with prions.5 This study analyzed the inhibition of PrPSc using 12 different derivatives of phenothiazine and acrimidine: Imipramine, Haloperidol, Carbamazepine, Clozapine, Phenazine, 2-Chlorophenothiazine, Phenothiazine, Promethazine, Promazine, Chlorprothixen, Chlorpromazine, and Acepromazine. Compounds were purchased from Sigma Aldrich and 9-substituted acridines were synthesized by reaction with 6,9-dichloro-2-methoxyacridine in phenol because the aliphatic side chains are important in mediating inhibition of prion formation. Neuroblastoma cells were infected with scrapie prions and subclones and each inhibitor was introduced into each cell line after culturing. Cells were lysed after 80% confluency, and cell lysates were digested with Proteinase K because those that develop beta sheet conformations are resistant to Proteinase K. This allowed the scientists to confirm that the cell lines only contained the aggregated or misfolded prions. SDS-PAGE and immunoblotting was performed according to standard techniques after centrifugation, and the immunoblots were incubated with a secondary antibody and developed with enhanced chemiluminescence (ECL). Densitometry was also performed.

The results from **Figure 1** indicated that, at concentrations ranging from 2-10 micromolars, treatment with promazine, chlorpromazine, and acepromazine led to the disappearance of PrPSc in treated cells after 6-day treatments. Addition of Dopamine to cultures of ScN2a cells in the presence of chlorpromazine did not reverse inhibition of PrPSc production. The tricyclic scaffold alone is not enough to inhibit the misfolded protein formation because phenazine, phenothiazine, and 2-chlorphenothiazine did not inhibit PrPSc formation. A side chain substituent on the central ring was necessary, as seen with the three derivatives that did produce inhibition.5

Because, as discussed briefly above, no promazine inhibitors have been studied that inhibit the prion effect found in alpha-synuclein, the aim of this study is to find such potent inhibitors that can cross the blood brain barrier, and resist this misfolding cascade that can lead to the formation of Lewy bodies in the substantia nigra and other regions of the brain, leading to and aiding in the progress of Parkinson’s Disease. The three main inhibitors analyzed in this study will be promazine, chlorpromazine, and acepromazine, all of which have been found to aid in preventing tumor growth for cancer therapies, and are able to cross the blood brain barrier in concentrations ranging from 2-10 micromolars.6

**Figure 1: PrPSc-inhibiting effects of phenothiazine derivatives and other psychopharmacological substances.** PrP immunoblots of protease-digested ScN2a cell lysates treated with respective inhibitor for 6 days. (Adapted from Ref 5)

1. **Experiment**

The aim of this experiment is to determine the potency of three different inhibitors on the prevention of aggregated alpha-synuclein (or prion like alpha synuclein formation) by finding the % Thioflavin T signal for each inhibitor at two different concentrations each (2 micromolar and 10 micromolar) at the beginning of the reaction between the inhibitors and the chronically aggregated alpha synuclein cell lines and after 30 minutes of incubation as well as analyzing molecular size markers found on SDS-PAGE gels taken after proteinase K digestion of the alpha-synuclein. For the inhibitors that are successfully able to resist alpha-synuclein aggregation, we expect the % ThT signal to be very low indicating a high level of inhibition as well as the presence of no molecular size markers that would indicate the presence of aggregated alpha-synuclein in SDS-PAGE gel results for these inhibitors.

II.a Protein Misfolding Cyclic Amplification (PMCA)

In order to analyze whether an inhibitor prevents the aggregation and misfolding of alpha-synuclein, we must first create a chronically aggregated alpha-synuclein cell line that can be used for inhibitor testing. Herva, Zibaee, et al. (2014) developed a method in which the protein misfolding cyclic amplification technique, used for amplifying prion aggregates, was adapted to grow alpha-synuclein aggregates.7 This study employed circular dichroism, electron microscopy, and SDS-PAGE to demonstrate the formation of alpha-synuclein. Results from this experiment are shown in **Figure 2** below.

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Object name is zbc0201481940001.jpgThe results from **Figure 2** show that PMCA causes aggregate formation of alpha-synuclein faster than non-PMCA incubation and shaking. Fibril formation was detected after 6 hours of PMCA with a max signal attained between 12 to 24 hours. This shows that the inhibitor study performed by this experiment will introduce inhibitors in that period from 12-24 hours after the PMCA reaction has been initiated in order to confirm that the alpha-synuclein will be at its maximal aggregated form.

**Figure 2: PMCA Analysis.** (A, C, D) growth kinetics of recombinant alpha-synuclein aggregates by PMCA versus non-PMCA samples. (B) ThT signal % for alpha-synuclein (non-PMCA samples, PMCA samples) and beta-synuclein (non-PMCA samples and PMCA samples). (Adapted from Ref 7)

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Object name is zbc0201481940003.jpgThis study also performed inhibition studies using Congo Red, Curcumin, etc. to determine if the PMCA technique was sensitive enough to use for inhibitor studies in the future. The SDS-PAGE gel results are shown in **Figure 3.** These results show that alpha-synuclein (whose control molecular size markers are shown on the last line of the gel) aggregation is clearly inhibited by Congo Red and Curcumin. The reason why these inhibitors would not be useful in treatment of Parkinson’s because Congo Red cannot pass through the blood brain barrier, and while Curcumin is able to cross through the barrier, it was found to be less potent in the face of promazine derivatives in inhibiting prion activity.5,6 As shown by results found in **Figure 3**, this PMCA technique is sensitive enough to be useful in inhibitor studies, and, therefore, will be very useful in this study.

**Figure 3: SDS-PAGE gel results of inhibitor analysis using PMCA technique to harvest chronically aggregated alpha-synuclein cell lines.** (Adapted by Ref 7)

II.b Promazine Derivatives

The three inhibitors that will be studied in this experiment are promazine, chlorpromazine, and acepromazine. These derivatives of promazine were found to be most potent in inhibiting prion activity in the study performed by Korth, May, et al. (2001) and they are able to cross the blood brain barrier. In order to find which concentration of these inhibitors would be most potent in inhibiting alpha-synuclein (assuming they do inhibit alpha-synuclein), we analyze two different concentrations of each inhibitor: one at 2 micromolars and one at 10 micromolars. These two concentrations were chosen due to that being the range in concentration that worked best in inhibiting prion activity in the study (Ref 5) that was discussed in detail in the introduction.5

Aside from these three main derivatives, there will be two control groups: one that contains just alpha-synuclein (in order to normalize the data generated) and one that contains just DMSO (in order to confirm that DMSO, the solution used to dilute the inhibitors, did not affect the alpha-synuclein PMCA).

II.c Introduction of Inhibitors to PMCA

PMCA alpha-synuclein will be established using standard protocols discussed by Herva, Zibaee, et al. (2014). Wild-type alpha-synuclein cell lines will be bought and purified using standard protocols.8 PMCA will be carried out by subjecting wild-type alpha-synuclein to repeated cycles of sonication and incubation after preparation of the alpha-synuclein via conversion buffer. The reaction mixtures will be performed in triplicate, and 2 microliters of appropriate inhibitor will be added to each reaction mixture (5 mixtures total in triplicates = 15 reaction mixtures).

5 microliters from each sample will be added to 495 microliters of Thioflavin T solution after the introduction of inhibitors and fluorescence will be measured using a spectrophotometer with 450 nm excitation and 480 nm emission settings (settings determined by study performed by Herva, Zibaee, et al. (2014))7. % Thioflavin Signal/Fluorescence will be analyzed in order to determine which inhibitors are potent in inhibiting aggregation of alpha-synuclein. Samples will be incubated for 30 minutes. After 30 minutes, another ThT Assay will be performed in order to compare with the fluorescence at the beginning of the reaction.

Proteinase K Digestion will be performed on a set of aliquots from each reaction mixture because alpha-synuclein that are aggregated will be resistant to Proteinase K digestion. This is because alpha-synuclein undergoes a conformational change when it misfolds in which it goes from an alpha-helix rich conformation to a beta-sheet rich conformation. Beta-sheet rich conformations are resistant to Proteinase K digestion. This will confirm that the protein we have in the aliquots is our protein of interest – aggregated alpha-synuclein.

SDS-PAGE will be performed on the aliquots after PK digestion in order to confirm results obtained from ThT Assay as well as reduce chance for artifact (which is why this is done after PK digestion instead of before PK digestion). Aliquots of alpha-synuclein PMCA and control samples will be mixed with loading buffer and low molecular mass standard protein ladders will be used as standard size markers. The low molecular mass standard is chosen because the aggregated alpha-synuclein will fall into this range of sizes. Gels will be stained with Coomassie Blue before being run and chemiluminescence will be induced by ECL reaction. Data will be collected.

The potency of the inhibitors analyzed will be confirmed by % ThT signals collected by the ThT assays performed at the beginning of the reaction and 30 minutes after incubation. The SDS-PAGE gel qualitative results obtained after PK digestion will help confirm or bolster these quantitative results obtained by the ThT assays.

1. **Discussion**

If the experiment follows the hypothesis that all three of the inhibitors will be successful in inhibiting alpha-synuclein aggregation, then the results will show that the % ThT signals will be low for all reaction mixtures in the assay done 30 minutes after incubation and the SDS-PAGE gel results will show no molecular size markers that indicate the presence of aggregated alpha-synuclein. From these results, I will be highly likely to draw a conclusion that the inhibitors do inhibit the aggregation of alpha-synuclein. However, there is also the possibility that these results will not be obtained, and, instead, there will be high % ThT signals and strong molecular size markers indicated that the aggregated alpha-synuclein still exists in the same concentration level as before. This would indicate that the inhibitors were unsuccessful in inhibiting the aggregation of alpha-synuclein.

A problem that may arise is that an assumption is made that only the aggregated alpha-synuclein will be resistant to PK digestion, leading to decreased artifact in the SDS-PAGE results. This is not necessarily true because although the aggregated alpha-synuclein is rich in beta sheets and will be resistant to the PK digestion, there may be artifacts (other compounds that find their way into the gel) that are also rich in beta-sheets that will pass through that “preliminary test”, leading to artifact present on the SDS-PAGE gel results. However, this will change the conclusion very minimally as measures are taken to get both quantitative and qualitative data (% ThT signal and SDS-PAGE gel chemiluminescence).

Once these inhibitors are analyzed with this study to determine if they are successful in resisting aggregation of alpha-synuclein, then future studies can be performed to better understand the mechanisms behind the inhibition. This can be done by Transmission Electron Microscopy or other forms of immunofluorescence or X-Ray Crystallography images can be taken of the aggregated alpha-synuclein and inhibitor reaction as time progresses. The images can be analyzed like a comic strip to see the mechanism occur first hand, if possible. These methods may or may not be viable. Regardless, the future studies that can be performed after this one will have the common goal of determining the mechanism behind which this inhibition occurs.

1. **References**

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