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Development Of A High-Throughput System For Screening Of Anti-Prion Molecules

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DEVELOPMENT OF A HIGH-THROUGHPUT SYSTEM FOR SCREENING OF ANTI-PRION MOLECULES

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DEVELOPMENT OF A HIGH-THROUGHPUT SYSTEM FOR SCREENING OF ANTI-PRION MOLECULES

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The misfolded prion protein causes and transmits disease in both humans and animals. As other infectious agents, prions display strain variation, which can generate different pathological outcomes in affected individuals. Unfortunately, there are no known therapies for these diseases, which at present are invariably fatal. In this work, the Protein Misfolding Cyclic Amplification technology (PMCA, an *in vitro* test that replicates minimum quantities of infectious prions) has been modified to screen for small molecules inhibiting prion protein misfolding in a strain-specific manner. In order to approach a high-throughput PMCA system, technical aspects in PMCA has been optimized for application of prions from laboratory rodents (i.e., mouse and Syrian hamsters) using a 96-well plate PMCA (96wp-PMCA) platform. Utilizing the 96wp-PMCA technique, a small number of anti-prion and anti-amyloid molecules has been tested against these prion strains using different solvents and at varying concentrations. My results show that regardless of sequence homology, prion strains are differentially responsive to known protein misfolding inhibitors. Continual optimization of PMCA towards a high-throughput system may be used not only for screening therapeutic agents but also for diagnosis.

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INTRODUCTION

The cellular prion protein (PrP^C) is naturally found in humans and animals (1). This protein can be found in a wide variety of tissues throughout the body. Specifically, it is found in higher concentrations in the brain, spinal cord, and lymphoid tissues (2, 3). From the cellular perspective, PrP^C is located on the cell surface (4). Currently, the function of this protein in physiological processes is unclear (5). However, proposed functions associated with PrP^C include protection of neurons from injury (6, 7), formation of synapses (8), or transport of copper into the cell (9–11). PrP^C has also been identified as a possible receptor for amyloid-β (Aβ) oligomers which appear to be the main pathological effectors in Alzheimer's disease (AD). However, based on studies showing that mice lacking the cellular prion protein display little or no abnormalities (12), it can be concluded that its functionality is not considered to be vital for cellular homeostasis or that its loss can be compensated for by other components. Under certain circumstances, PrP^C can misfold into a disease-causing form referred to as PrP^{Sc} (13). The length of PrP^{Sc} and its amino acid composition are identical to PrP^C. The difference between both entities lies in the secondary structure of the protein, with PrP^{Sc} consisting of more β-pleated sheets in comparison to α-helices found in PrP^C (1, 14). Notably, the misfolded form of the prion protein is known to cause and transmit a group of disorders termed as Transmissible Spongiform Encephalopathies (TSEs, (15, 16)).

Prion diseases have been identified in several mammalian species, including humans (17). Human prion diseases can be classified into three categories: acquired, familial, and sporadic (18). Infection as a cause of human TSEs accounts for approximately one percent of cases (WHO). In these cases, infectious prions have been described as being introduced to humans through cannibalism (19), medical procedures

(20–22), and ingestion of beef products derived from cattle afflicted by bovine spongiform encephalopathy (23). Human exposure to these materials leads to diseases known as Kuru (24), iatrogenic Creutzfeldt-Jakob Disease (iCJD, (25)), and variant CJD (vCJD, (23, 26, 27)), respectively. Five to ten percent of cases can be classified as familial or hereditary (WHO). In these cases, patients with CJD, Gerstmann Straussler-Scheinker (GSS), and Fatal Familial Insomnia (FFI) can be found to have point mutations in the gene encoding the prion protein, resulting in accelerated misfolding and stabilization of the disease-causing PrP isoforms (28, 29). Similarly, as with other neurodegenerative diseases, the majority of human prion diseases are identified to be sporadic. Patients affected by sporadic cases are not infected or have a family history of the disease. This latter group includes sporadic CJD (sCJD, (30)), sporadic fatal insomnia ((31), and variable protease sensitive prionopathy (VPSPr, (32)). The events triggering the first misfolded process in sporadic TSEs are unknown, and likely occur in a stochastic manner (30). Prion diseases are not selective towards humans and affect animals as well. In fact, the frequency of prion diseases in animals is significantly higher (33). There are a few types of animal prion diseases that can be considered prevalent. These include scrapie affecting sheep and goats (34), bovine spongiform encephalopathy affecting cows (35, 36), and chronic wasting disease (CWD) affecting cervid (37). All these TSEs have been found to exist in farm settings; however, only CWD has been identified in the wild as well (38–40). Due to the epidemic associated with CWD and its unclear zoonotic potential, this prion disease has recently gained research and public interest (41). Currently, efforts are being made by the scientific community and regulatory agencies to avoid unpleasant scenarios as the BSE epidemic that was experienced at the end of the last century (42). When an infected animal is identified in a farm setting, all animals that inhabit the same area are sacrificed in order to prevent the spreading of the disease. The

presence of an infectious animal in the wild can transport the disease across a large geographical distance.

Both humans and animals affected by prion diseases develop unique pathological features as assessed by *post mortem* examination of brain and some peripheral tissues (43). These include spongiform degeneration (44), accumulation of disease-associated prion conformers (PrP^{Sc}) (45), loss of function and death of neurons (13), microglial activation (45), and astrocytes proliferation (46). Importantly, the clinical and pathological manifestation of TSEs within and across species is diverse. Compelling evidence has suggested that prion diseases originate from specific variants or "strains" of PrP^{Sc} (47). It is speculated that these different variants are a result of distinctive tertiary and quaternary conformations that PrP^{Sc} acquires (48). From a diagnosis standpoint, these strains may have specific biochemical properties that facilitate their identification and diagnosis (47). These biochemical characteristics, such as glycosylation pattern and structure of the protein, are distinctive of strains behaving differently. This can pose an obstacle in the pursuit of treatments against these diseases.

Currently, there are no cures or treatments against any type of prion disease in both humans and animals. Different laboratories have attempted to prevent or delay the onset of the disease. Most of these strategies have been assessed using *in vitro* assays or animal models of TSEs (49, 50). Unfortunately, the majority of therapeutic approaches have achieved modest effect through the extension of the incubation period for the disease (51, 52). Therapeutic strategies explored against TSEs can be organized broadly into three categories: (i) treatment-based approaches (gene therapy, immunizations, manipulations at the immune system level, and cell-based therapies), (ii) small molecules (altering either prion replication or cell signaling cascades), and (iii) prion competition treatment. Some of these experimental approaches have been seen to delay

onset of the disease (53), act as a prophylaxis through protection from the disease (54–56), or suppress prion replication (57). Unfortunately, the majority of these treatments have only been tested against a select number of strains. Predominantly, these strains belong to laboratory adapted rodent prion strains and not strains present naturally in humans or animals. Due to the proposition that these strains are a result of different conformations, an avenue that has shown promising results for one specific prion strain may not be proficient against a second one (58).

To study prion diseases in the laboratory setting, animal models such as mice and Syrian hamsters have been utilized (47). Unfortunately, there is a high monetary cost associated with these models due to their long incubation periods for disease manifestation (being as long as 450 days). Because of the need to test a large array of compounds for activity against prion replication, this experimental format is unviable. The Protein Misfolding Cyclic Amplification (PMCA) technique has been shown to replicate conformational properties of infectious prions while maintaining their biologically relevant features (59). This technique allows for the production of a large amount of the infectious prion protein (PrP^{Sc}) at the expense of PrP^{C} through multiple cycles consisting of incubation and sonication (Figure 1). The incubation periods allow for the recruitment and conversion of PrP^{C} to PrP^{Sc} which adds to the PrP^{Sc} aggregates. In order to expose more free ends of the aggregates to aid in the conversion process, sonication causes the aggregate to be broken. Due to its sensitivity and simplicity, PMCA can be used for several purposes, including: screening of biological samples (60), modeling of prion strains and the species barrier phenomena (61), effectiveness of different strategies to remove prions from biological samples (62), diagnosis of human and animal prion diseases (63–65)), and many others.

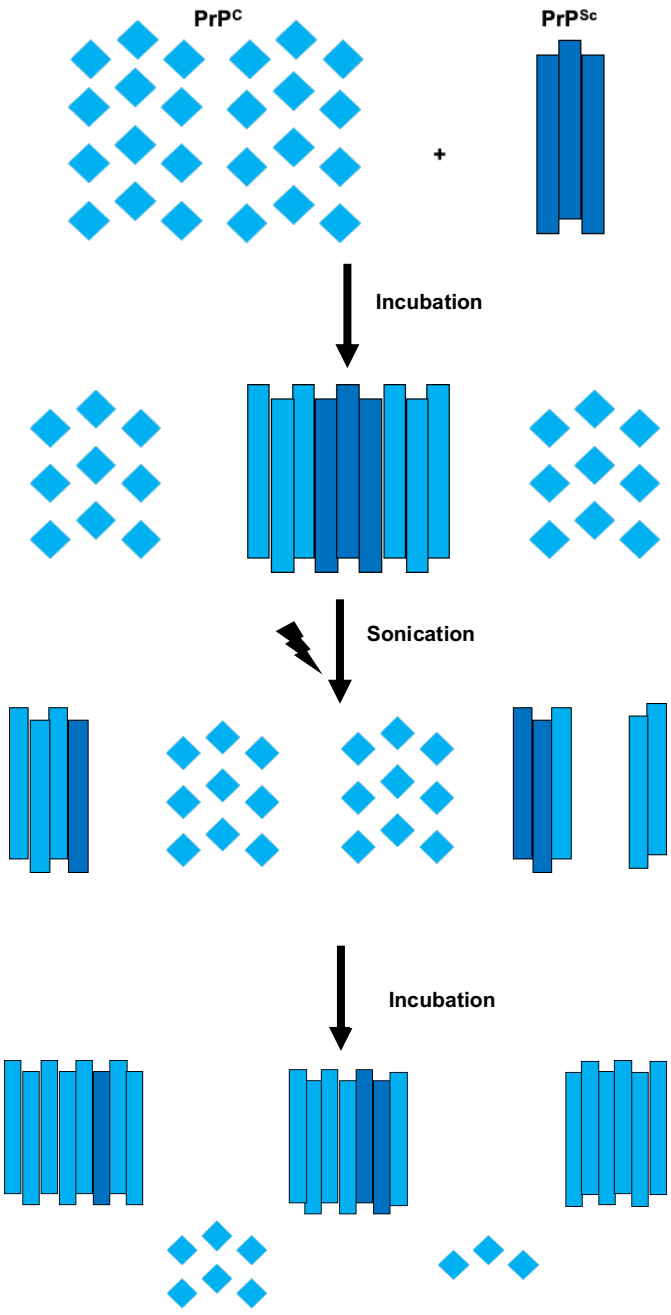


Figure 1. Graphical representation for protein misfolding cyclic amplification. The *in vitro* technique is based on the nucleation polymerization principle of protein aggregation. The PrP^{Sc} aggregates recruits and converts PrP^{C} during the incubation period, and sonication allows for more free ends available to aid in the conversion process. Multiple cycles of incubation and sonication occur during the PMCA process.

For this master's thesis, I focused on establishing a protocol that increases the actual throughput of the PMCA technology with the goal to screen a small selection of known anti-prion and anti-amyloid molecules. Six rodent prion strains associated with mice and hamsters were chosen for this project. The mouse prion strains included the Rocky Mountain Laboratory Strain (RML, (66, 67)), 301C (68), and ME7 (69–71) (originated from scrapie, bovine spongiform encephalopathy, and scrapie, respectively). Hamster prions comprised HY (72), SSLOW (73), and 263K (74) (with sources of scrapie, *in vitro* misfolded recombinant PrP, and transmissible mink encephalopathy, respectively). I chose these strains because they have been readily used to model and study prion diseases in a laboratory setting and their distinct biochemical (and presumably structural) features. My working hypothesis is that by modification of PMCA for high-throughput screening of small molecules, I will find strain specific anti-prion agents.

In order to utilize the PMCA technique to screen small molecules, I modified PMCA for a 96-well plate (96wp) format for different prion strains. Modifications to different parameters, including, but not limited to, composition of the PMCA conversion buffer, alterations on incubation and sonication periods and sonication power, and signal analysis performed via dot blotting, which is in considerably faster when compared to currently used western blots, were made in order to standardize 96wp-PMCA. I aimed to decrease the reagents needed and the time required for PMCA in order to advance the technique in a high-throughput format.

After the standardization of 96wp-PMCA, I used this technique to screen a small selection of molecules, which possess proven anti-prion capabilities in different systems (mostly cell-based). Anti-prion molecules and anti-amyloid molecules were evaluated against six rodent prion strains. The anti-prion molecules have been tested against a

selected number of prion strains belonging to human and animals (49, 75) while the anti-amyloid molecules are compounds which have been shown to inhibit the replication of non-prion amyloids (76).

From these aims, with the standardization of 96wp-PMCA and its application for the screening of small molecules, I completed the main goal of identifying small molecules able to inhibit the *in vitro* prion misfolding process against different strains. The long-term goal will be for these identified molecules to be tested in animal models, and prospectively utilized in clinical trials. Additionally, this technology may be optimized to further increase its throughput and identify novel strain-specific inhibitors. In addition, the standardized principles of the 96wp-PMCA platform could be applied towards other diseases involving the accumulation of misfolded proteins, such as, Alzheimer's and Parkinson's disease. The PMCA technique can be further modified and optimized in order to attain a high-throughput screening system.

MATERIALS AND METHODS

Preparation of Brain Tissue

Wild-Type (WT) mouse or hamster brains were collected from animals euthanized by CO_2 inhalation based on University and Federal guidelines. After euthanasia, animals were subjected to cardiac perfusion. Perfusion buffer was created by adding 5 mM EDTA (Fisher Scientific Company PR-V4231) to 1X PBS (Fisher Scientific Company SH30256LS). Each animal was manually perfused with 30 mL of perfusion buffer. After perfusion, brains were removed, rinsed in perfusion buffer, frozen in liquid nitrogen and stored at -80°C. Prion-Infected mice or hamsters were euthanized by CO_2 inhalation once

reaching advanced clinical stages of prion disease as previously described (77, 78). Collected brains were stored at -80°C until used.

Preparation of PMCA Substrate

Conversion Buffer (CB) was made consisting of: 150 mM NaCl (Sigma Aldrich S3014), 1% volume/volume (v/v) Triton X-100 (Sigma Aldrich T9284) and 1X PBS, pH 7.0-7.3. Before use, 50 mL of CB was supplemented with a complete protease inhibitor (PI) cocktail tablet containing EDTA (Thermo Fisher Scientific PIA32965). Perfused wild-type brains were weighed and combined with conversion buffer (CB) containing PI to generate a 10% weight/volume (w/v) homogenate that was prepared in ice-cooled Potter-Elvehjem tissue grinders. Homogenates were centrifuged at 800 x g at 4°C for 45 s. Pellets were disposed of while supernatants were well-mixed by vortexing and then placed on ice. Supernatants were aliquoted, frozen in liquid nitrogen, and stored at -80°C. All the procedures mentioned above were performed using prion-free facilities and equipment. These aliquots, which are the final PMCA substrate, are referred to as normal brain homogenate (NBH).

Preparation of Inoculum

The protocol is similar to the substrate preparation aside from the following: homogenization buffer was prepared by mixing one complete PI tablet with EDTA in 1X PBS. The prion-infected brain was homogenized in 10% w/v, as described above and centrifuged. Supernatants were mixed, aliquoted and stored at -80°C until use.

96-well plate PMCA (96wp-PMCA)

PMCA protocol was extensively modified from previously described procedures (79). Two teflon beads (PTFE Grade Balls 3/32) (Hoover Precision Products) were added to each well of 96-well plates (Thomas Scientific C18096). Prions were added to each well in amounts varying from 1 x 10^{-2} to 1 x 10^{-5} % w/v brain homogenate equivalent by dilution in NBH. Each reaction was performed in a total volume of 50 µL. Samples were covered with twelve strip lids (Thomas Scientific C18096-C) and plates were placed in the horn of a Qsonica Q700 automatic sonicator system. The overall length of each passage was between 24 and 48 hrs. Each PMCA cycle consisted of continuous cycles of 29 min and 40 s of incubation followed by 20 s of sonication. During the PMCA procedure, the sonicator horn is inside an incubator set at 37°C. PMCA reactions were additionally supplemented with different concentrations of ethanol or dimethyl sulfoxide, or small molecules dissolved in these solvents (small molecule concentrations varying from 100 µM to 0.1 µM). Negative controls consisted of unseeded PMCA reactions.

Analysis of 96wp-PMCA Products

Each well from 96wp-PMCA was treated with proteinase K (PK) (Sigma Aldrich P2308) at a 100 µg/mL final concentration. After PK was added to the wells, the plate was placed in an Eppendorf Thermomixer for 1 hr at 37°C set to perform 450 rpm. To stop the PK reaction, 5 mM of phenylmethane sulfonyl fluoride (PMSF) (Fischer Scientific Company AC215740050) was added to each well and incubated at 70°C for 10 min. Samples were then assessed for the presence of PK-resistant PrP either by western blot or dot blot. For dot blots, Whatman Gel Block Paper (GE Healthcare Life Science 10427812) was placed onto a Bio-Dot Apparatus (Bio-rad 1706455) followed by a nitrocellulose membrane

(Fischer Scientific Company 45-004-003). From each well, 5 µL of content was placed directly onto the nitrocellulose membrane. Membranes were dried using a blow dryer and then transferred into an incubation chamber containing 10 mL of 3M guanidinium thiocyanate (Fischer Scientific 50-491-761) for 10 min. Later, membranes were rinsed three times with 50 mL of washing buffer (0.05% Tween in 1X PBS) and then transferred to a 10% blocking solution consisting of 5% w/v dry nonfat milk (Fischer Scientific Company NC9952266) dissolved in washing buffer. Membrane blocking lasted 1 hr and was performed by placing the membrane on a rocking platform at room temperature. The membrane was later incubated with monoclonal 6D11 anti-prion antibody (Santa Cruz Biotechnology sc-58581) at a 1:10,000 dilution in 5% w/v blocking buffer for 1 hr on a rocking platform at room temperature followed by 3 washes (10 min, 5 min, and 5 min) with washing buffer. Membranes were then incubated with a horseradish peroxidase-linked polyclonal antibody mouse IgG (Sigma Aldrich A5906) at a dilution of 1:3,000 in 5% w/v blocking buffer for 1 hr on a rocking platform at room temperature. The membrane was again washed for 10 min, 5 min, and 5 min with washing buffer and developed using an ECL system following manufacturer's recommendations (Fisher Scientific 45-002-401). For western blots, a similar procedure was performed, with the difference that PK digested samples were previously fractionated in SDS-PAGE and transferred to nitrocellulose membranes as described (79).

RESULTS

Optimization of Parameters for Dot Blot Analysis

Conventional PMCA procedure classically involves detection of PrP^{Sc} signals by western blots (79). The utilization of conventional PMCA could be an option for the screening of small molecules. However, I chose to optimize conventional PMCA for a 96-

well plate format because the amount of time involved could be drastically reduced by the use of an alternative detection method, increasing the throughput and number of samples to be analyzed. I chose the dot blot method due to its proven efficiency for prion detection (80), the reduced time of this technique compared to western blots, and the reduced volume of sample needed to obtain clear readouts. Aside from alterations to the PMCA process, analyzing the samples with dot blots should reduce analysis times from two days to one day compared to currently used western blots.

In order to achieve a high-throughput PMCA screening system in the future through further modifications, the levels of samples for the assay and detection should be reduced. In that sense, the minimum volume of PMCA products to be used in dot blots must be determined. Importantly, positive readings obtained at the selected volume should be homogeneous across the plate. For that purpose, I used a 10% w/v brain homogenate from a mouse infected with the RML prion strain. Three different volumes were utilized, including 3 µL, 5 µL, and 7 µL (Figure 2). These volumes are substantially lower than the 20-30 µL regularly utilized in western blot detection (79). Ninety-six aliquots of the same brain preparation were placed directly on the nitrocellulose membrane. Because the samples used consisted of concentrated infectious material, I expected that positive signals should be detected throughout the entire membrane. All dot blots in this project were evaluated in an all-or-none fashion. These membranes did not contain negative control wells in order to assess for uniform signal across the plate. With only 3 µL used, signal was not uniformly detected for all the samples (Figure 2A). On the contrary, both the volumes of 5 µL and 7 µL generated uniform results across the plates. These experiments were performed in duplicates with reproducible outcomes. I determined the optimal minimum volume of the samples required for analysis to be 5 µL due to the desire to use the lowest sample volume possible.

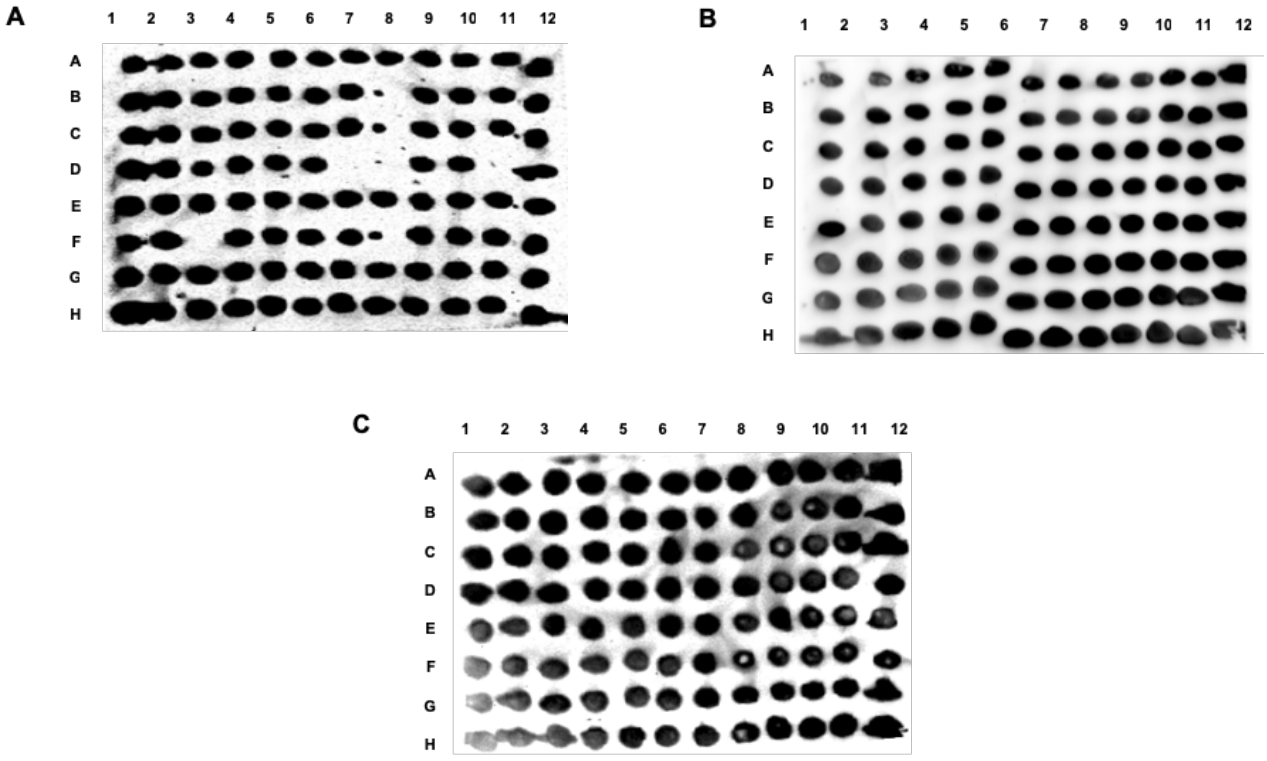


Figure 2. Effects of sample volume utilized in dot blot analysis. Three different volumes (3μL (A), 5μL (B), and 7μL (C)) of 10% weight/volume (w/v) brain homogenate prepared from the brain of a terminally ill mouse infected with the Rocky Mountain Laboratory (RML) prion strain were directly placed on nitrocellulose membranes as described in Methods. These samples were not PK-treated.

In addition to the reduction in sample volume, processing time was decreased as well. A major time constraint in the classical PMCA setup is seen in the primary antibody incubation time. Based on current western blot protocols used to analyze PMCA products, the standardized time required for primary antibody incubation is sixteen hours (81, 82). Considering that several primary antibodies work at one hour of incubation (79, 83), I decided to try the same on my developing dot blot protocol. Similar to the experiments shown in Figure 2, I used 10% w/v brain homogenate from a terminally ill mouse brain infected with the RML prion strain as sample. Ninety-six aliquots of 5 µL each of this material were loaded directly on the nitrocellulose membrane. For comparison, two incubation times were tested (1 and 16 hrs, Figure 3). I concluded that one hour of primary antibody incubation was sufficient for the dot blot to yield positive signals in all wells, similar to what was observed by incubating the membrane with the conventional 16 hours. The reduction of the primary antibody incubation duration contributed to the decrease of time required for the analysis of the samples.

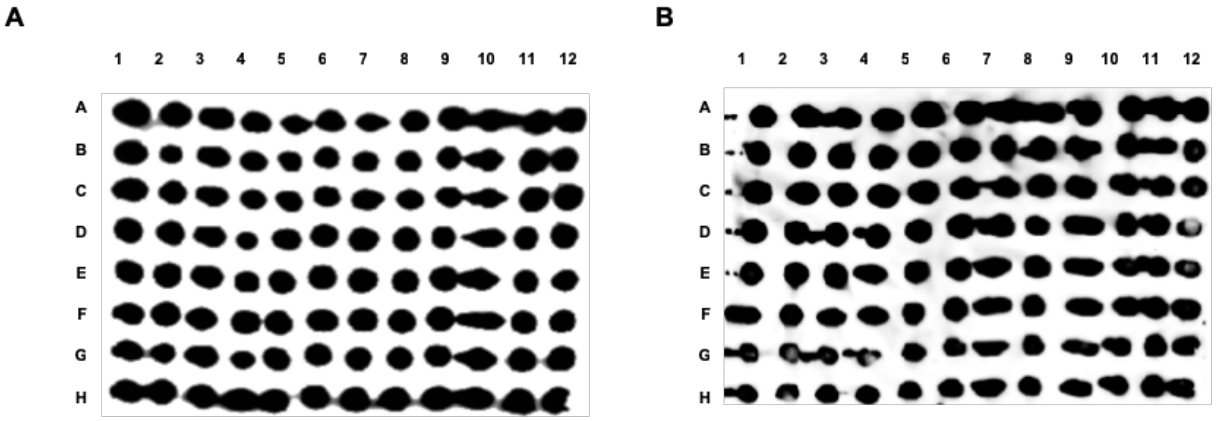


Figure 3. Reduction of primary antibody incubation times does not affect dot blot readout. 5 µL aliquots from a 10% weight/volume (w/v) brain homogenate prepared using a brain from terminally ill mice infected with the Rocky Mountain Laboratory (RML) prion strain were directly placed on nitrocellulose membranes. These samples were not PK-treated. Primary antibody (6D11) incubation was tested for two different durations, lasting either 1 hr (A) or 16 hrs (B).

Optimization of the 96wp-PMCA

The next step was to modify PMCA with the intention of optimizing it towards a high-throughput format. For that purpose, I modified this technique for its use in 96-well plates instead of currently used individual tubes. While using plates, I tested modifications of PMCA components such as passage duration, sample volume in each well, and specifications of the sonicator to accommodate for the number of samples. Current settings used in conventional PMCA may work differently in the 96 well plate format and should be tested. Based on previous research performed, the PMCA process may require supplementation in order to work efficiently (79). Ethylenediaminetetraacetic acid (EDTA) is a chelating agent that binds to divalent cations, particularly copper (II) ions in solution. Divalent cations have been shown to inhibit prion replication in PMCA (84, 85) Similar results have been observed for prions bound to metallic surfaces where the release of cations into the PMCA reaction are likely the cause for reduced or no replication ((86) and unpublished data). Due to the interaction that occurs between the prion protein and divalent ions, the usage of EDTA can prevent unwanted interactions and allow for PMCA to be more efficient. I tested two concentrations of EDTA in 96wp-PMCA, including 0 mM (using protease inhibitor that was EDTA-free) and 1 mM (using a complete protease inhibitor) supplemented into normal brain homogenate containing a $1x10^{-4}$ % w/v dilution of an RML-infected brain homogenate. Samples from both tested conditions were PK treated prior to dot blot analysis. My results showed no signal for samples in the EDTA-free context while in the presence of 1 mM EDTA uniform positive signals were seen (Figure 4A). I concluded that in the absence of EDTA, the PMCA process is hindered and 1 mM of EDTA is required for PMCA to work efficiently.

To further reduce the time required for HT-PMCA, I needed to reduce the number of incubation/sonication cycles. In traditional PMCA, multiple passages (rounds) each

involving several incubation/sonication cycles are required (79). Each PMCA round classically comprises durations between 24 hrs and 72 hrs. Extended times per PMCA round are required to obtain a uniform amplification throughout all samples placed in the sonication horn (79). For my assay, I decided to test 24 and 48 hrs in a single PMCA round. I observed comparable results for both times tested and selected the one of 24 hrs cycle time for further assays (Figure 4B).

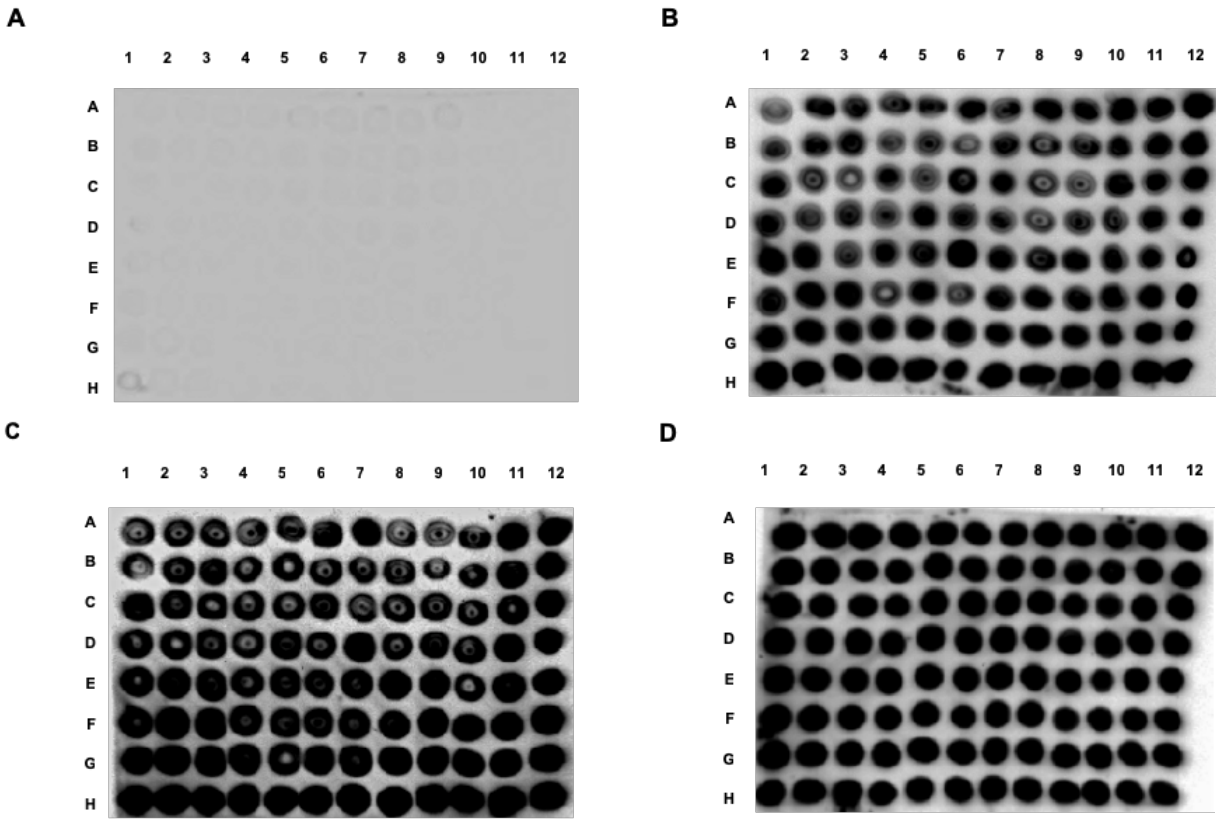


Figure 4. Effects of EDTA supplementation and PMCA passage duration in 96wp-PMCA. Modifications to optimize PMCA for a 96 well-plate format included changes at the conversion buffer and incubation/sonication levels. (i) Normal wild-type brain homogenate (PMCA substrate) was subjected to 0 mM (A) or 1mM (B) of EDTA supplementation in conversion buffer used to homogenized samples. Normal brain homogenate was utilized to make a 1 x 10^{-4} % w/v RML brain homogenate dilution and subjected to one PMCA passage of 24 hrs. (ii) Similar samples as described in (A) (EDTA supplemented) were subjected to PMCA passages of 24 hrs (C) or 48 hrs (D).

As previously stated, the amount of PrP^{Sc} seeds in the PMCA reaction was another component which required optimization in order to approach this technique for a high-throughput format. Therefore, it was essential to determine the lowest dilution that can be utilized without jeopardizing uniform amplification of prions across all positions in the 96-well plate. The main purpose of manipulating PMCA for a 96-well plate was to provide a new avenue to use the technique for the screening of small molecules; therefore, the lower dilution would be beneficial to maximize the probability of identifying prospective molecules while also isolating efficient drugs. I tested, in my developing 96wp-PMCA assay, four concentrations of RML prions in regards of the dilution of an infected brain homogenate: 1×10^{-2}, 1×10^{-3}, 1×10^{-4} and 1×10^{-5} % w/v (Figure 5). PMCA products derived from each of the four dilutions were PK-treated and analyzed. Uniform amplification can be seen at all four concentrations. When the dilution of 10^{-5} was tested against the other prion strains of interest, I was unable to detect uniform amplification of the samples (not shown). After testing the dilution of 1×10^{-4} % w/v for other strains, I was able to conclude that the appropriate dilution across multiple strains would be 1×10^{-4}% w/v.

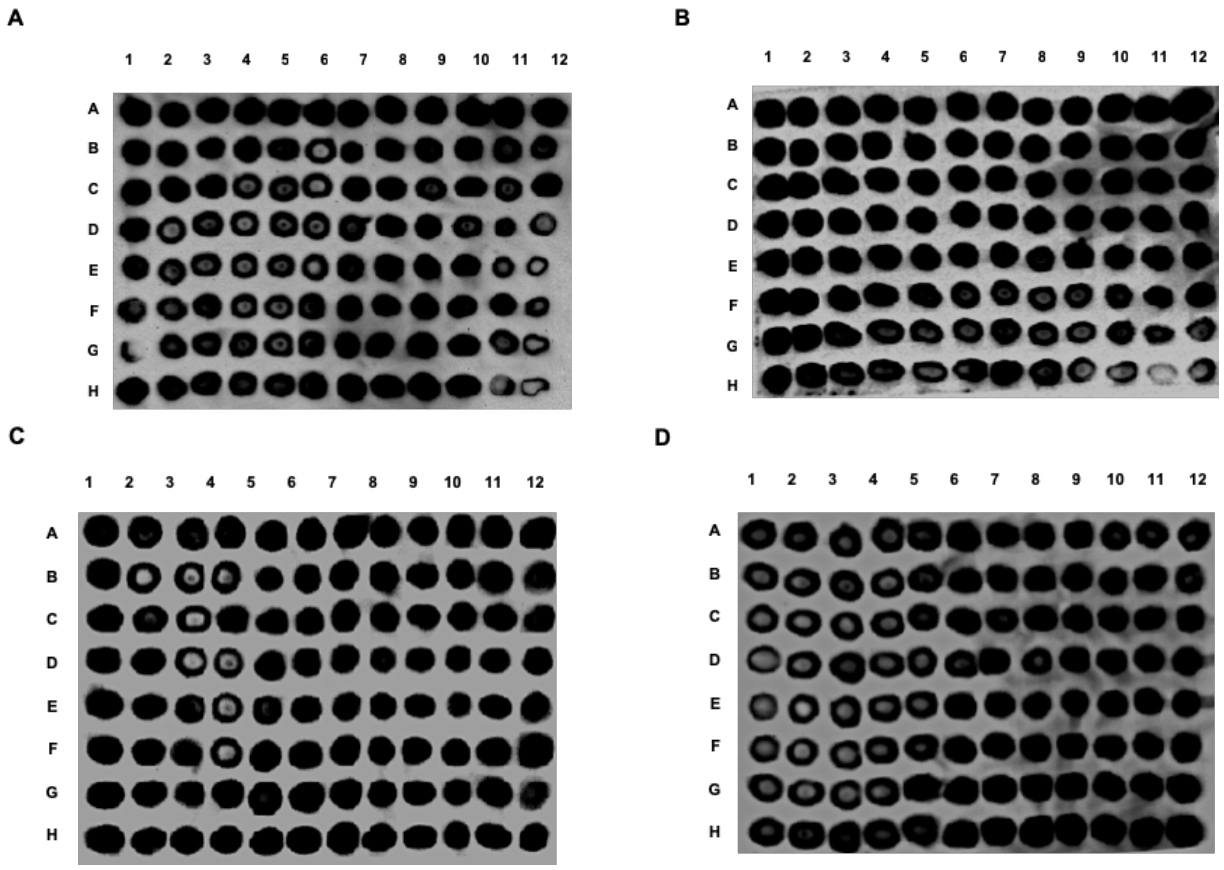


Figure 5. Effects of varying PrP^{Sc} dilutions in 96wp-PMCA performance. Varying dilutions of RML brain homogenates (1×10^{-2} (A), 1×10^{-3} (B), 1×10^{-4} (C), and 1×10^{-5} (D) % w/v) were tested for detection using 96wp-PMCA for 24 hrs. All PMCA products were PK treated before analysis through dot blots.

In summary, my modifications to use PMCA in a 96 well plate format included the supplementation of 1 mM EDTA into the normal brain homogenate (Figure 4A) and supplementation of seeds at a 1 x 10^{-4} % w/v dilution of infected brain homogenate (Figure 5B). These conditions fulfilled my requirements of uniform amplification across the 96 well plate for multiple rodent prion strains. Aside from alterations to the actual PMCA process, I made other changes including the utilization of two teflon beads each in comparison to three teflon beads, and reduction of the overall volume in well from 90 µL to 50 µL. Comprehensively, these changes allow for PMCA to be modified for a scheme that is more optimal for the screening of small molecules targeting the prion misfolding process. A summary of the changes implemented for the 96wp-PMCA compared to conventional PMCA are listed in Table 1.

Table 1. Comparison of PMCA and 96wp-PMCA

	PMCA	96wp-PMCA
Platform	PCR Tubes	96-Well Plate
Volume	100 µL	50 µL
# of Teflon Beads	3 Beads	2 Beads
Number of Sonication Passages	3 Passages	1 Passage
Sonication Time	6 Days	1 Day
Volume of Sample Used for Analysis	19µL	5µL
Signal Analysis	Western Blot	Dot Blot
Total Time	8 Days	2 days

Standardization of Experimental Prion Strains

Based on the parameters established above, I tested the *in vitro* amplification performance of six rodent prion strains. Specifically, I used three mouse-derived prion strains (RML (66, 67), 301C (68), and ME7 (69–71)) and three hamster-derived prion strains (HY (72), SSLOW (73), 263K (74)). All these strains originated from different sources, including sheep (RML, ME7), goat (263K), mink (HY), bovine (301C) prions, or synthetically derived (SSLOW). The Rocky Mountain Laboratory (RML) strain was chosen because several compounds halting its replication have been identified (49). The other strains were chosen based on their suspected different conformations. As mentioned, my working hypothesis is anti-prion molecules will have different responses depending on the prion strain that they are tested against. Each of these six strains were subjected to 24 hours of 96wp-PMCA at a 1 x 10^{-4} % w/v brain dilution of seeds. Additionally, 4-6 negative control (non-seeded) samples were included to assess for possible cross-contamination during PMCA and handling of samples. After PMCA, all samples were PK-treated to remove non-misfolded PrP and 5 µL from each well were dispensed onto nitrocellulose membranes for analysis. I observed uniform amplification for the mouse and hamster prion strains (Figure 6 and Figure 7, respectively). Negative control samples resulted in no signal, as expected. I further confirmed the presence of bona-fide PrP^{Sc} by western blot (not shown).

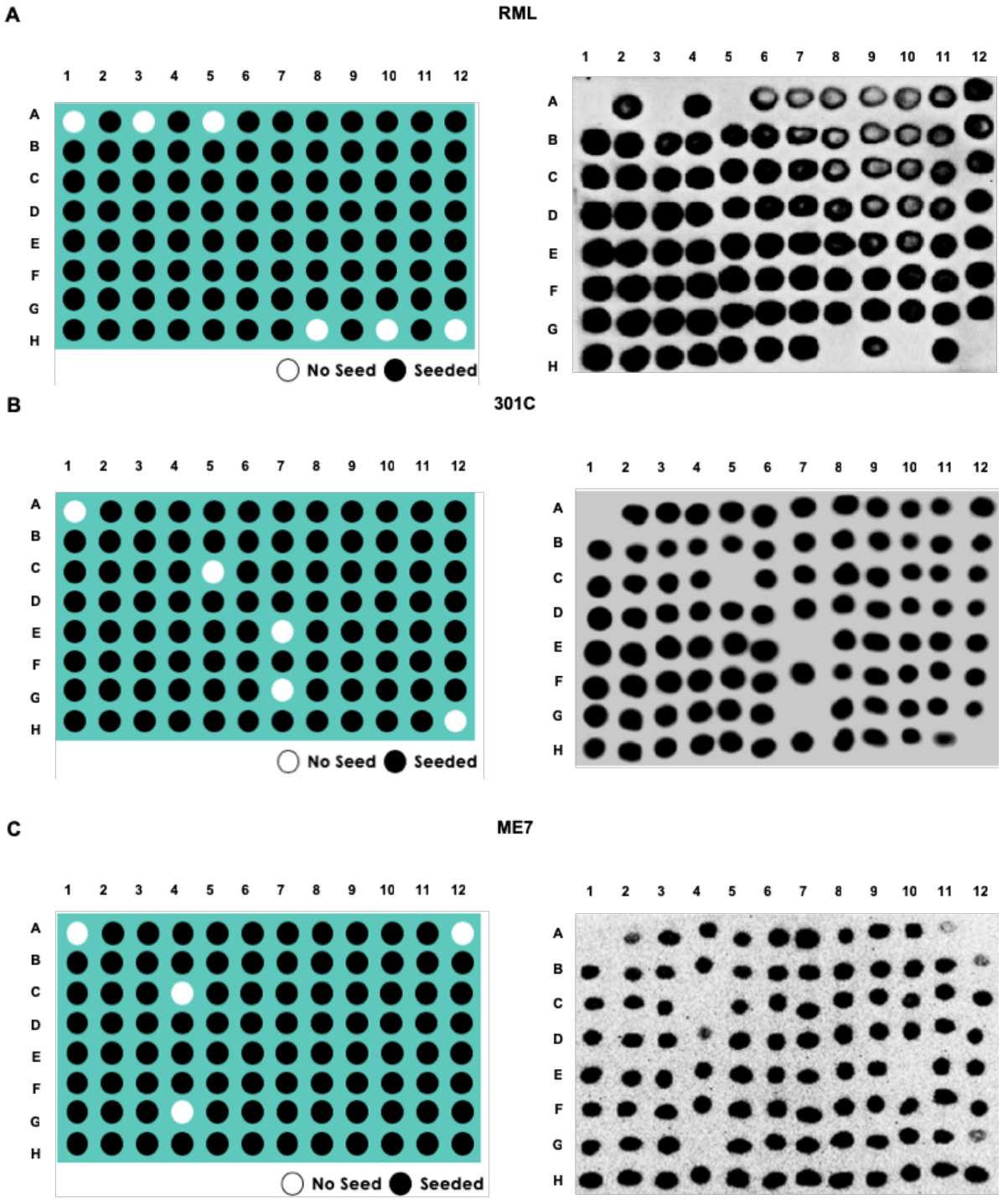
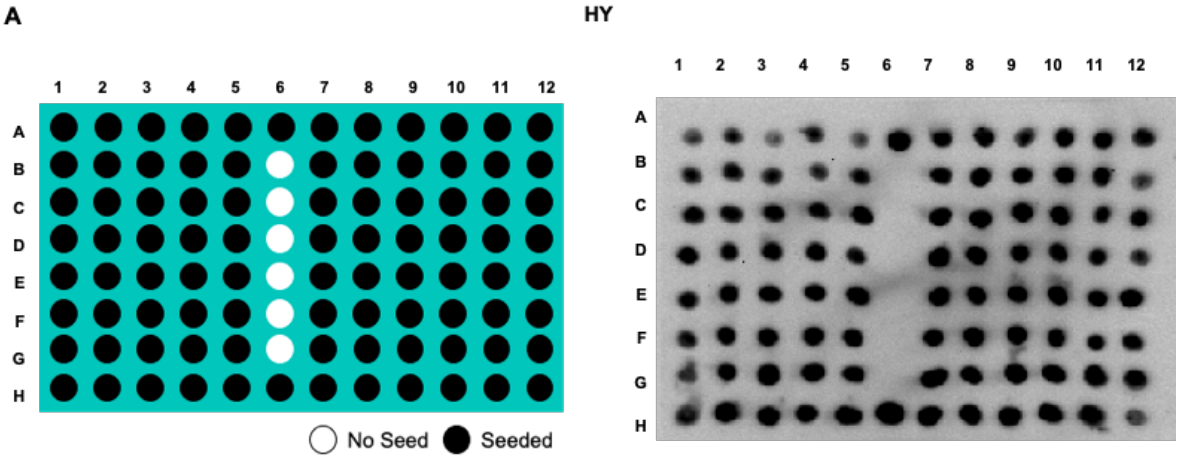
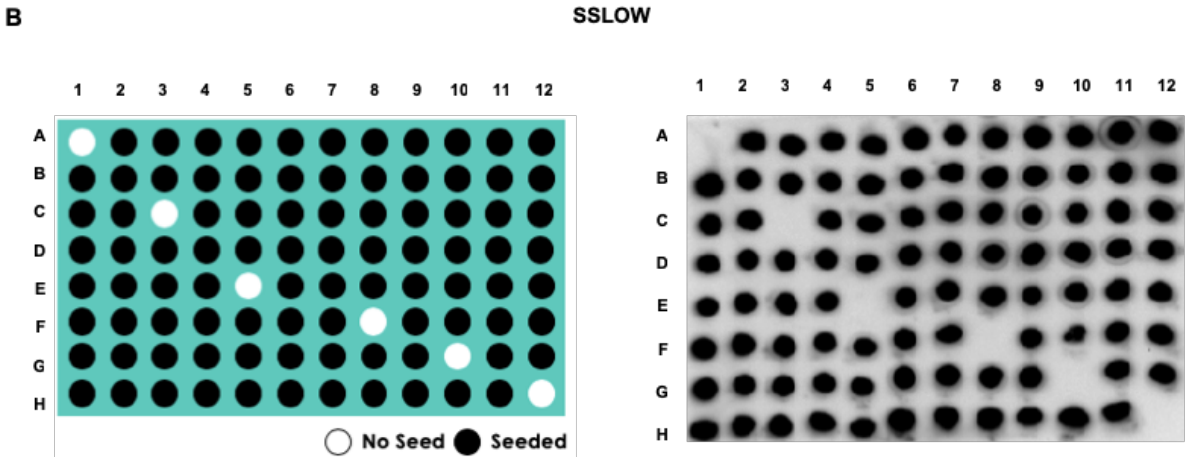

A
RML
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
No Seed
Seeded
B
301C
C
ME7

Figure 6. Standardized 96wp-PMCA for three mouse prion strains. From top to bottom, 10^{-4} dilutions of RML (A), 301C (B), and ME7 (C) prions, respectively, were subjected to 24 hrs of 96wp-PMCA. Each plate contained 4-6 negative control wells as depicted in white on the corresponding diagram (left panels). In these control samples, no PrP^{Sc} added as described in Methods.


A
HY
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
No Seed
Seeded


B
SSLOW
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
No Seed
Seeded

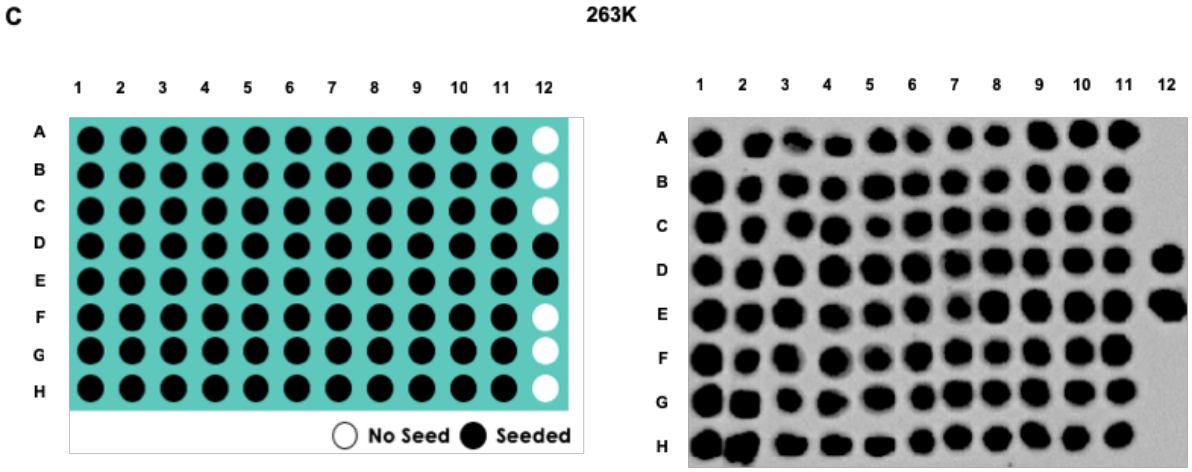

C
263K
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
No Seed
Seeded

Figure 7. Standardized 96wp-PMCA for three hamster prion strains. From top to bottom, 1 x 10^{-4} % w/v dilutions of HY (A), SSLOW (B), and 263K (C) prions, respectively, were subjected to 24 hrs of 96wp-PMCA. Each plate contained 4-6 negative control wells as depicted in white on the corresponding diagram. In these control samples, no PrP^{Sc} added as described in Methods.

Due to the intention of testing a wide range of molecules, and in order to account for molecule solubility in varying solvents, I tested the effect of dimethyl sulfoxide (DMSO) and Ethanol (EtOH) on 96wp-PMCA performance. This experiment was necessary in order to determine that the effects seen in the presence of the tested compounds were not due to the solvents in which they were dissolved. Plates were set up with two strains from each animal species, specifically RML and 301C for mice and HY and 263K for hamsters. For all prion strains, a 1:2 dilution gradient was set up for each of the solvents (starting from 8% v/v of solvent in each case). As seen in Figure 8, amplification of the mouse prion strains displayed inhibition only at 8% EtOH but no inhibition at any of the DMSO concentrations tested (Figure 8A). For hamster prions, inhibition of 263K replication was seen at 8% ethanol, while HY displayed inhibition with EtOH at 8% and 4% (Figure 8B). Overall, these data suggest that PMCA is impacted at these specific concentrations; therefore, molecules should be dissolved in concentrations where inhibition is not seen. I therefore dissolved all compounds in 1% v/v of solvent and tested their effects on *in vitro* prion replication using my 96wp-PMCA format.

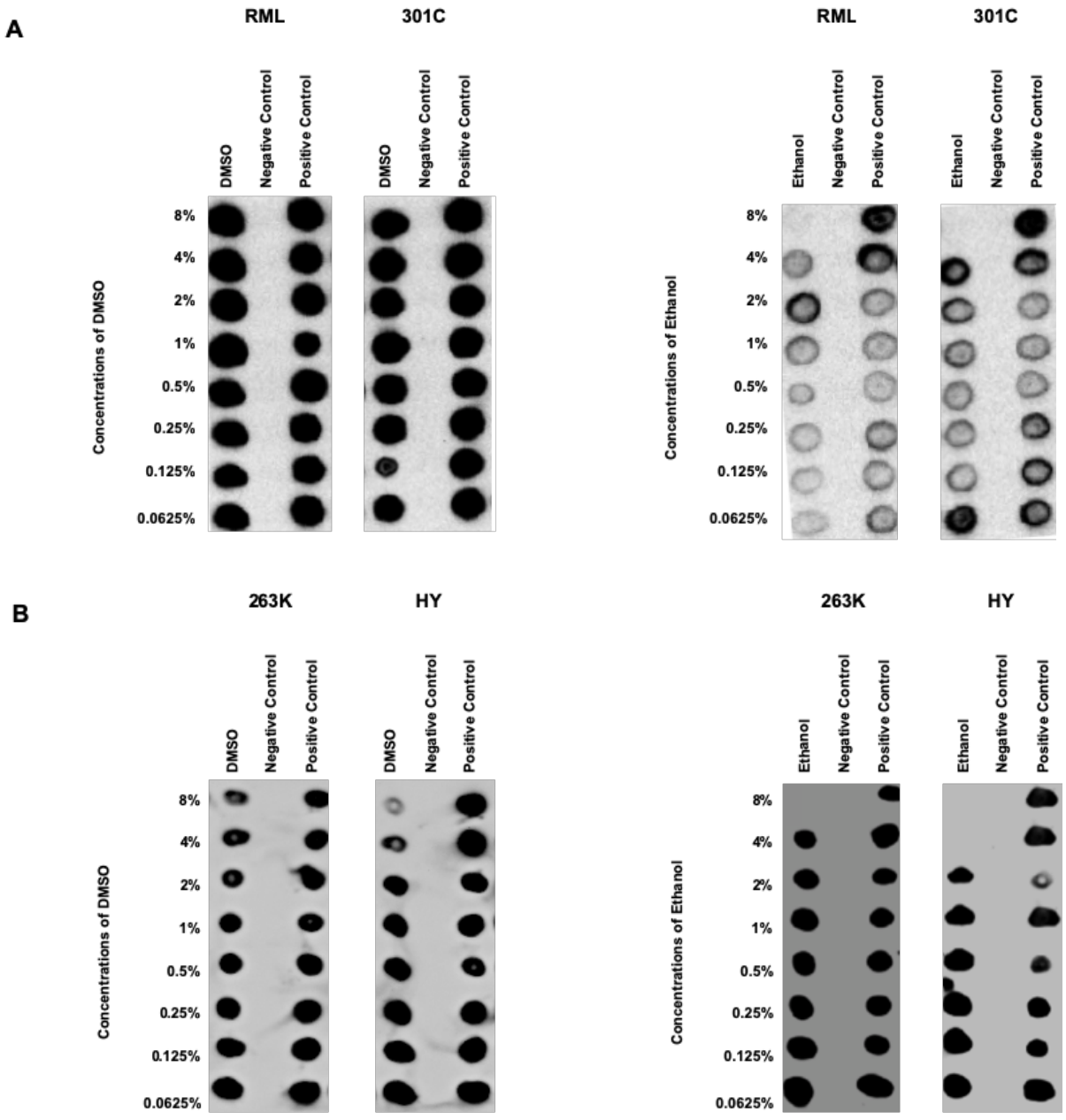


Figure 8. Effects of drug solvents in 96wp-PMCA. Prospective solvents, DMSO and ethanol, were tested at eight different concentrations (ranging from 8% to 0.0625%) against two mouse strains (A) and two hamster strains (B).

Evaluation of compounds in 96wp-PMCA

Next, the optimized 96wp-PMCA set up was used to test the effect of a small selection of known anti-prion (Figure 9) and anti-amyloid (Figure 12) molecules against the six prion strains previously shown to replicate in this format. The majority of the anti-prion molecules were chosen due to their previously reported effectiveness against RML prions in a cell-based *in vitro* assay (49). The six anti-amyloid molecules chosen in this thesis work have been previously tested for their activity against misfolded proteins involved in Alzheimer's and Parkinson's Disease (76, 87, 88). Importantly, the anti-prion activity of the latter set of compounds has not been studied. All compounds were dissolved, based on their solubility, in either 1% v/v DMSO or 1% v/v ethanol. In the appropriate solvents, four concentrations (100 μM, 10 μM, 1 μM, and 0.1 μM) of each molecule were used to supplement 96wp-PMCA reactions. The total volume of each well after the addition of the molecules was 50 μL as described in my previous optimization of the assay. Each 96-well plate also contained proper controls, including negative control samples and seeded wells supplemented with solvents only. To assess for reproducibility, each condition was tested in triplicates (each replicate in a different plate).

The mouse strains (RML, 301C, and ME7) were tested against ten anti-prion molecules; astemizole, Congo red, curcumin, quinacrine dihydrochloride, imatinib, resveratrol, tannic acid, tetracycline hydrochloride, thioflavin T, and tetrandrine (Figure 10). Each of these molecules have been tested against a select number of strains for its inhibitory effects in varying models. From the ten molecules tested, inhibition in prion replication was observed only with three of the them (Congo red, tannic acid, and thioflavin T). Although the same three molecules were effective against all three mouse strains, a difference in lowest concentration of inhibition was seen for two of them: Congo red and thioflavin T. For 301C prions (Figure 10B), both of these molecules displayed

inhibition at 10 μM. This was different from the 100 μM effective concentration observed for RML prions (Figure 10A). In comparison to the other two mouse prion strains, ME7 (Figure 10C) was only inhibited by tannic acid. When these anti-prion molecules were tested against the hamster prion strains (HY, SSLOW, and 263K, Figure 11), Congo red, tannic acid, and thioflavin T caused inhibition of these strains as well. In addition to those three molecules, curcumin was shown to inhibit HY at 100 μM when dissolved in EtOH (Figure 11A). This molecule also inhibited 263K misfolding at 100 μM in both solvents (Figure 11C). Table 2 and Table 3 summarize the lowest concentration of compounds capable of inhibiting *in vitro* replication on each prion strain. These results support my hypothesis that the effectiveness of molecules with anti-prion activity may vary depending on the prion strain that is tested.

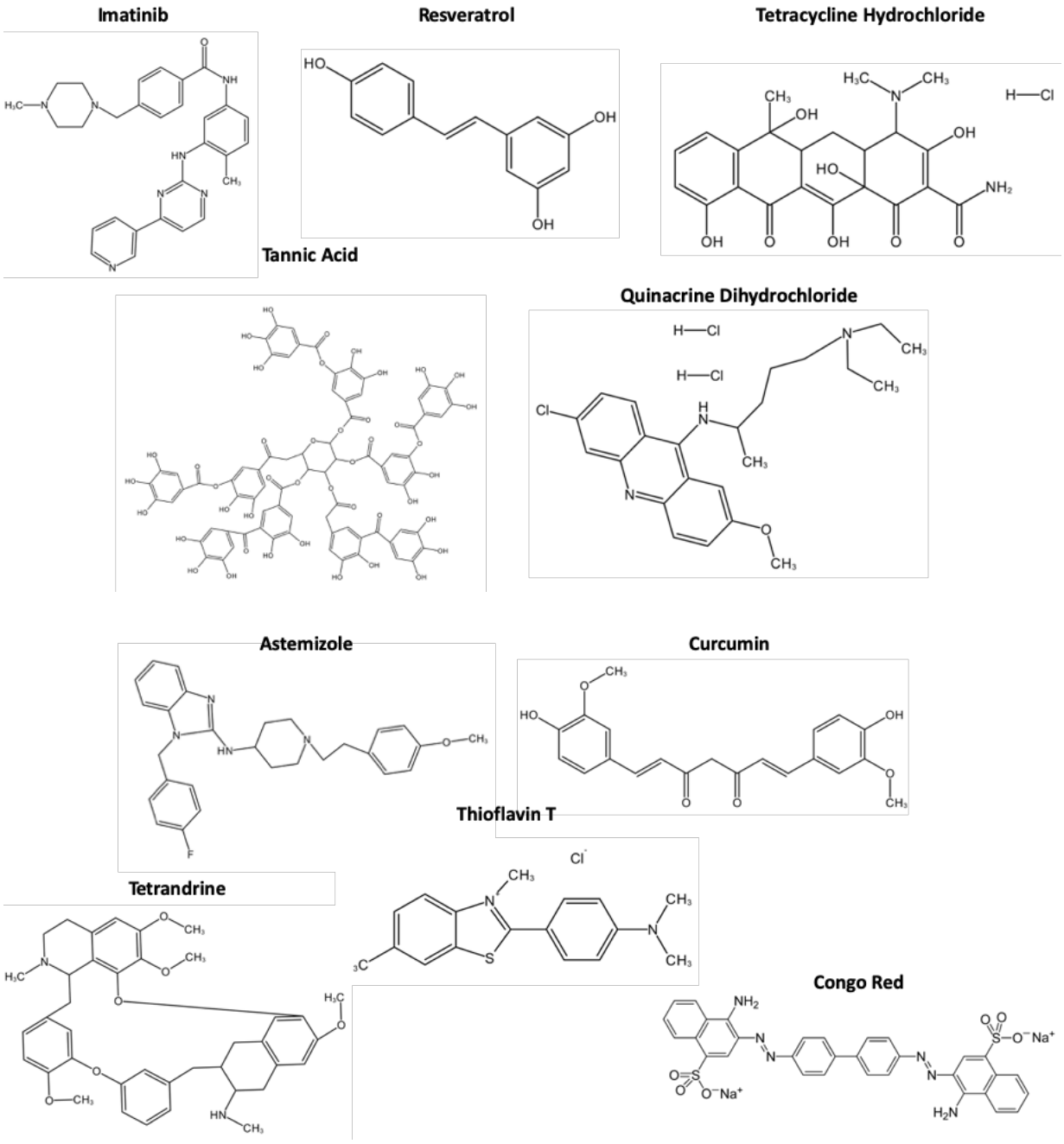


Figure 9. Selection of anti-prion molecules. Ten known anti-prion compounds were chosen to be evaluated for inhibitory effects in 96wp-PMCA against rodent prion strains.

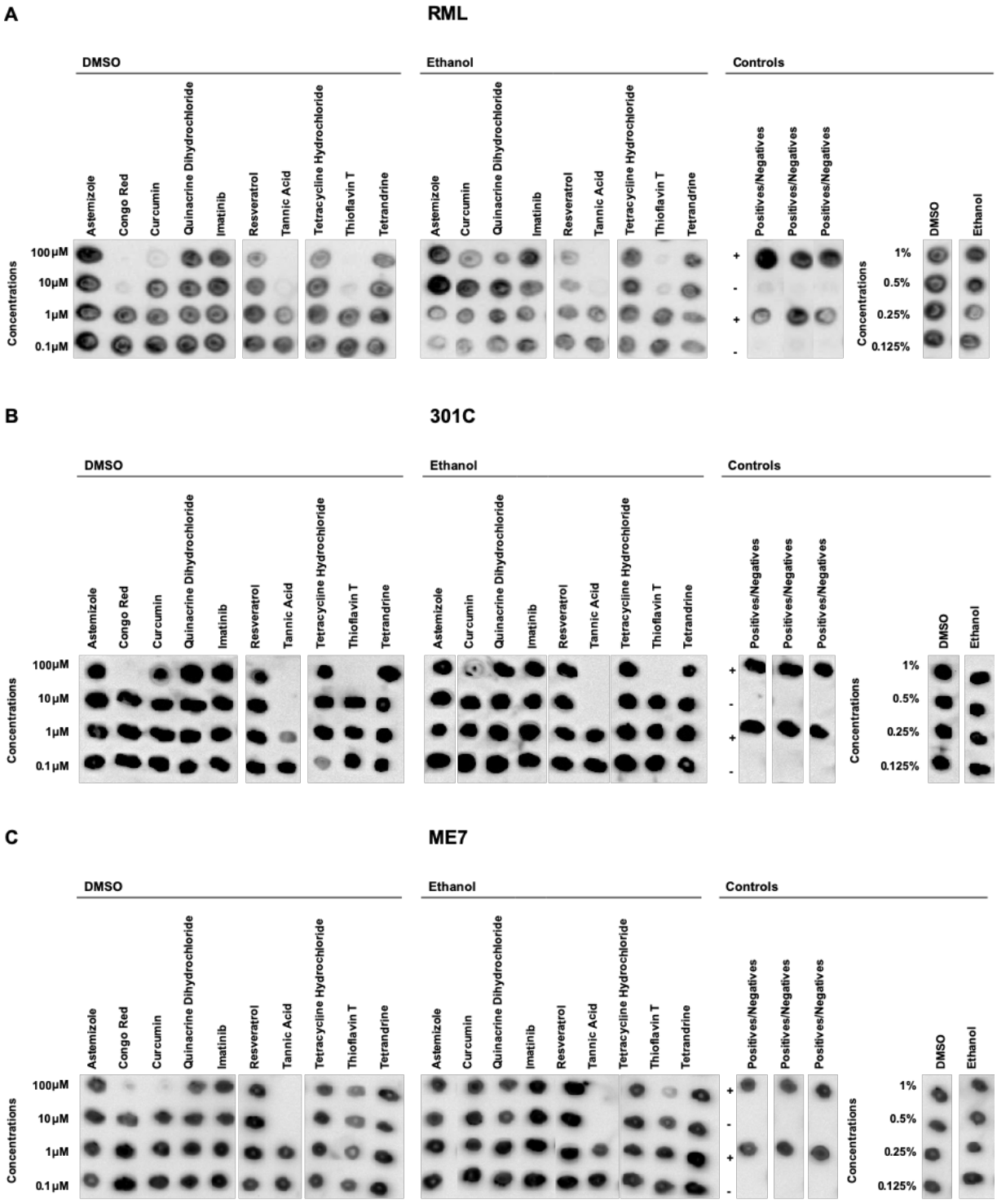

A
RML
DMSO
Ethanol
Controls
Astemizole
Congo Red
Curcumin
Quinacrine Dihydrochloride
Imatinib
Resveratrol
Tannic Acid
Tetracycline Hydrochloride
Thioflavin T
Tetrandrine
Positives/Negatives
Concentrations
100µM
10µM
1µM
0.1µM
1%
0.5%
0.25%
0.125%
DMSO
Ethanol
B
301C
C
ME7

Figure 10. Evaluation of known anti-prion small molecules in 96wp-PMCA (mouse prion strains). Ten known anti-prion compounds were tested at four different concentrations in either DMSO or EtOH for their activity against RML (A), 301C (B), and ME7 (C) *in vitro* replication. Each molecule was tested in concentrations ranging from 100 μM to 0.1 μM. Controls for solvents and positive samples and negative samples without compounds were included to monitor the assay. Dot blots were modified for labeling. Figure is representative from three independent assays.

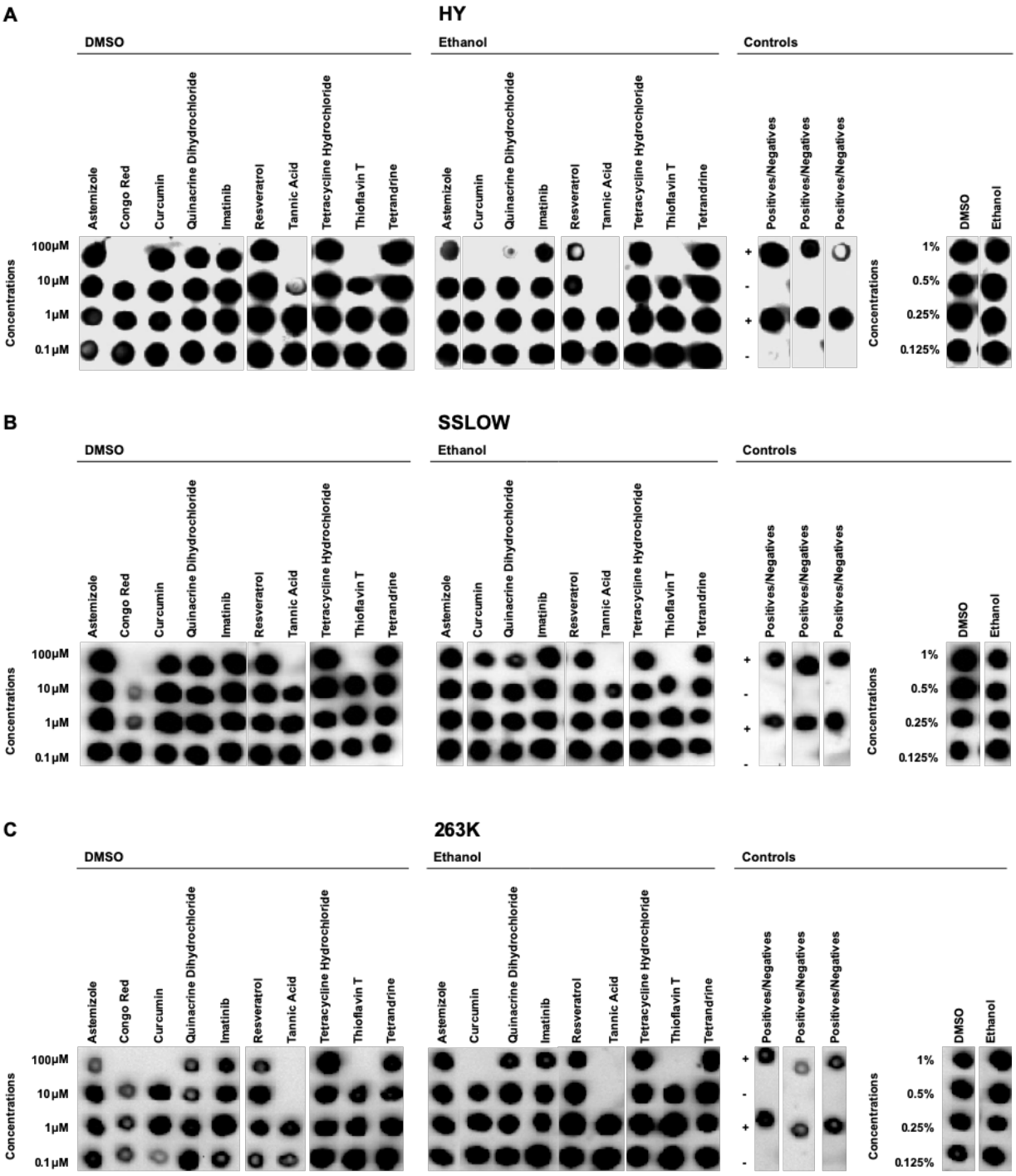

A
HY
DMSO
Ethanol
Controls
Astemizole
Congo Red
Curcumin
Quinacrine Dihydrochloride
Imatinib
Resveratrol
Tannic Acid
Tetracycline Hydrochloride
Thioflavin T
Tetrandrine
Positives/Negatives
Concentrations
100µM
10µM
1µM
0.1µM
1%
0.5%
0.25%
0.125%
DMSO
Ethanol
B
SSLOW
C
263K

Figure 11. Evaluation of known anti-prion small molecules in 96wp-PMCA (hamster prion strains). Ten known anti-prion compounds were tested at four different concentrations in either DMSO or EtOH for their activity against HY (A), SSLOW (B), and 263K (C) *in vitro* replication. Each molecule was tested in concentrations ranging from 100 μM to 0.1 μM. Controls for solvents and positive samples and negative samples without compounds were included to monitor the assay. Dot blots were modified for labeling. Figure is representative from three independent assays.

Table 2. Lowest Inhibition Concentration for Mouse Prion Strains

		RML		301C		ME7	
		DMSO	Ethanol	DMSO	Ethanol	DMSO	Ethanol
Anti-Prion Molecules	Astemizole	No Inhibition	No Inhibition	No Inhibition	No Inhibition	No Inhibition	No Inhibition
	Congo Red	10µM	N/A	100µM	N/A	No Inhibition	N/A
	Curcumin	No Inhibition	No Inhibition	No Inhibition	No Inhibition	No Inhibition	No Inhibition
	Quinacrine Dihydrochloride	No Inhibition	No Inhibition	No Inhibition	No Inhibition	No Inhibition	No Inhibition
	Imatinib	No Inhibition	No Inhibition	No Inhibition	No Inhibition	No Inhibition	No Inhibition
	Resveratrol	No Inhibition	No Inhibition	No Inhibition	No Inhibition	No Inhibition	No Inhibition
	Tannic Acid	10µM	10µM	10µM	10µM	10µM	10µM
	Tetracycline Hydrochloride	No Inhibition	No Inhibition	No Inhibition	No Inhibition	No Inhibition	No Inhibition
	Thioflavin T	10µM	10µM	100µM	100µM	No Inhibition	No Inhibition
	Tetrandrine	No Inhibition	No Inhibition	No Inhibition	No Inhibition	No Inhibition	No Inhibition
Anti-Amyloid Molecules	Azure A	10µM	10µM	10µM	10µM	100µM	100µM
	Azure B	1µM	1µM	10µM	10µM	10µM	10µM
	Azure C	10µM	10µM	10µM	10µM	1µM	1µM
	Quinacrine Mustard Dihydrochloride	No Inhibition	No Inhibition	No Inhibition	No Inhibition	No Inhibition	No Inhibition
	Rhodanine	No Inhibition	No Inhibition	No Inhibition	No Inhibition	No Inhibition	No Inhibition
	Thionin Acetate Salt	100µM	100µM	10µM	100µM	10µM	10µM

Table 3: Lowest Inhibitor Concentrations for Hamster Strains

		HY		SSLOW		263K	
		DMSO	Ethanol	DMSO	Ethanol	DMSO	Ethanol
Anti-Prion Molecules	Astemizole	No Inhibition	No Inhibition	No Inhibition	No Inhibition	No Inhibition	No Inhibition
	Congo Red	100µM	N/A	100µM	N/A	100µM	N/A
	Curcumin	No Inhibition	100µM	No Inhibition	No Inhibition	100µM	100µM
	Quinacrine Dihydrochloride	No Inhibition	No Inhibition	No Inhibition	No Inhibition	No Inhibition	No Inhibition
	Imatinib	No Inhibition	No Inhibition	No Inhibition	No Inhibition	No Inhibition	No Inhibition
	Resveratrol	No Inhibition	No Inhibition	No Inhibition	No Inhibition	No Inhibition	No Inhibition
	Tannic Acid	100µM	10µM	10µM	10µM	10µM	10µM
	Tetracycline Hydrochloride	No Inhibition	No Inhibition	No Inhibition	No Inhibition	No Inhibition	No Inhibition
	Thioflavin T	100µM	100µM	100µM	100µM	100µM	100µM
	Tetrandrine	No Inhibition	No Inhibition	No Inhibition	No Inhibition	No Inhibition	No Inhibition
Anti-Amyloid Molecules	Azure A	10µM	10µM	100µM	100µM	10µM	10µM
	Azure B	10µM	10µM	10µM	10µM	10µM	10µM
	Azure C	1µM	1µM	10µM	10µM	10µM	10µM
	Quinacrine Mustard Dihydrochloride	100µM	100µM	100µM	100µM	100µM	100µM
	Rhodanine	No Inhibition	No Inhibition	No Inhibition	No Inhibition	No Inhibition	No Inhibition
	Thionin Acetate Salt	10µM	10µM	100µM	100µM	100µM	100µM

The replication of mouse prion strains (RML, 301C, and ME7) was also tested in the presence of six molecules; azure A, azure B, azure C, quinacrine mustard dihydrochloride, rhodanine, and thionin acetate salt, proven to be active against other amyloidogenic proteins (76) (Figure 13). Assays were performed in a similar fashion as depicted in Figure 10. From the six tested molecules, four of them inhibited replication for all mouse prion variants (azure A, azure B, azure C, and thionin acetate). Specifically, azure B and azure C were effective at 1 μM against RML (Figure 13A) and ME7 (Figure 13C). The four molecules that displayed inhibition did so at different concentrations that were dependent on the prion strains they were evaluated against. In a similar trend to the anti-prion molecules, I am able to see strain specific patterns of inhibition for the anti-amyloid molecules as well. In addition to the four molecules that were effective against mouse strains, quinacrine mustard showed clear anti-prion activity against all the hamster prions tested in this project (Figure 14). Although inhibition was detected across the three hamster prion strains, it is important to acknowledge that effect occurred only at the highest concentration tested (100 μM). The molecule displaying the lowest anti-prion activity against hamster prions was azure C (effective at 1 μM against HY prions) (Figure 14A). Table 3 summarizes the lowest concentration displaying anti-prion activity each hamster prion strain for both anti-prion and anti-amyloid molecules. When evaluating the results across both species and both groups of molecules, it becomes evident that there may be a prion strain selective pattern of activity. Depending on the strain that the molecule was tested against and the solvent, a selection of the compounds was discovered to have distinct inhibition concentrations.

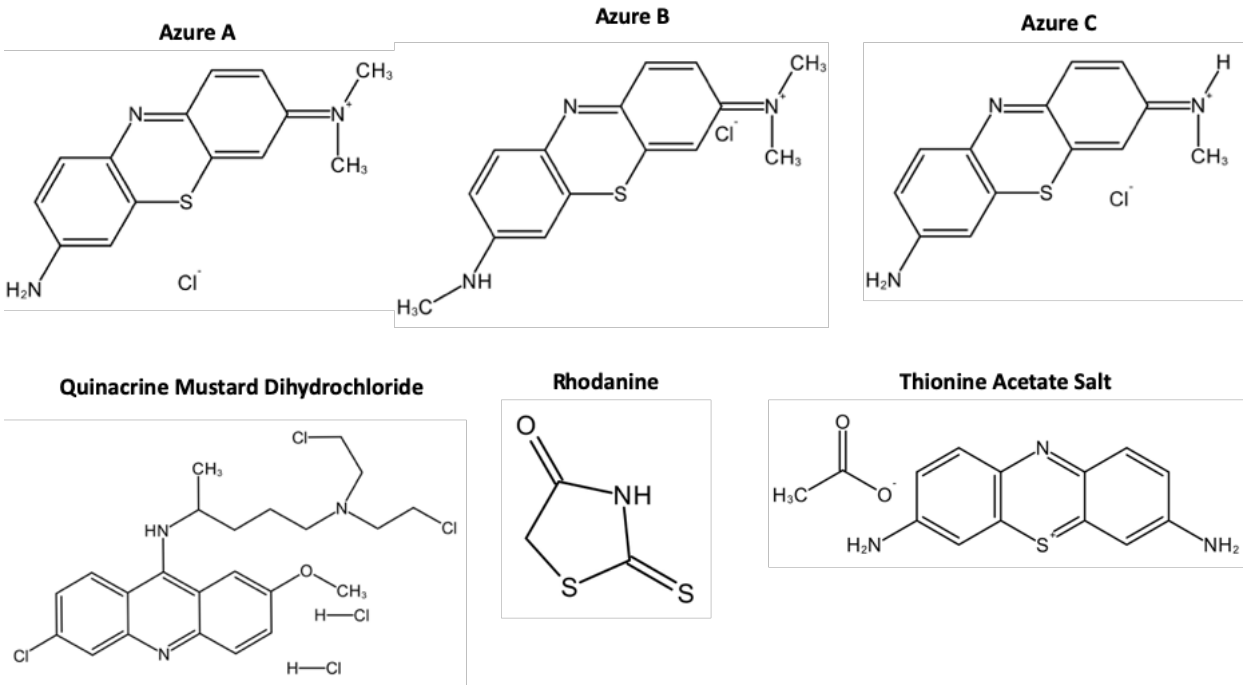


Figure 12. Selection of anti-amyloid molecules. Six known anti-prion compounds were chosen to be evaluated for inhibitory effects in 96wp-PMCA against rodent prion strains.

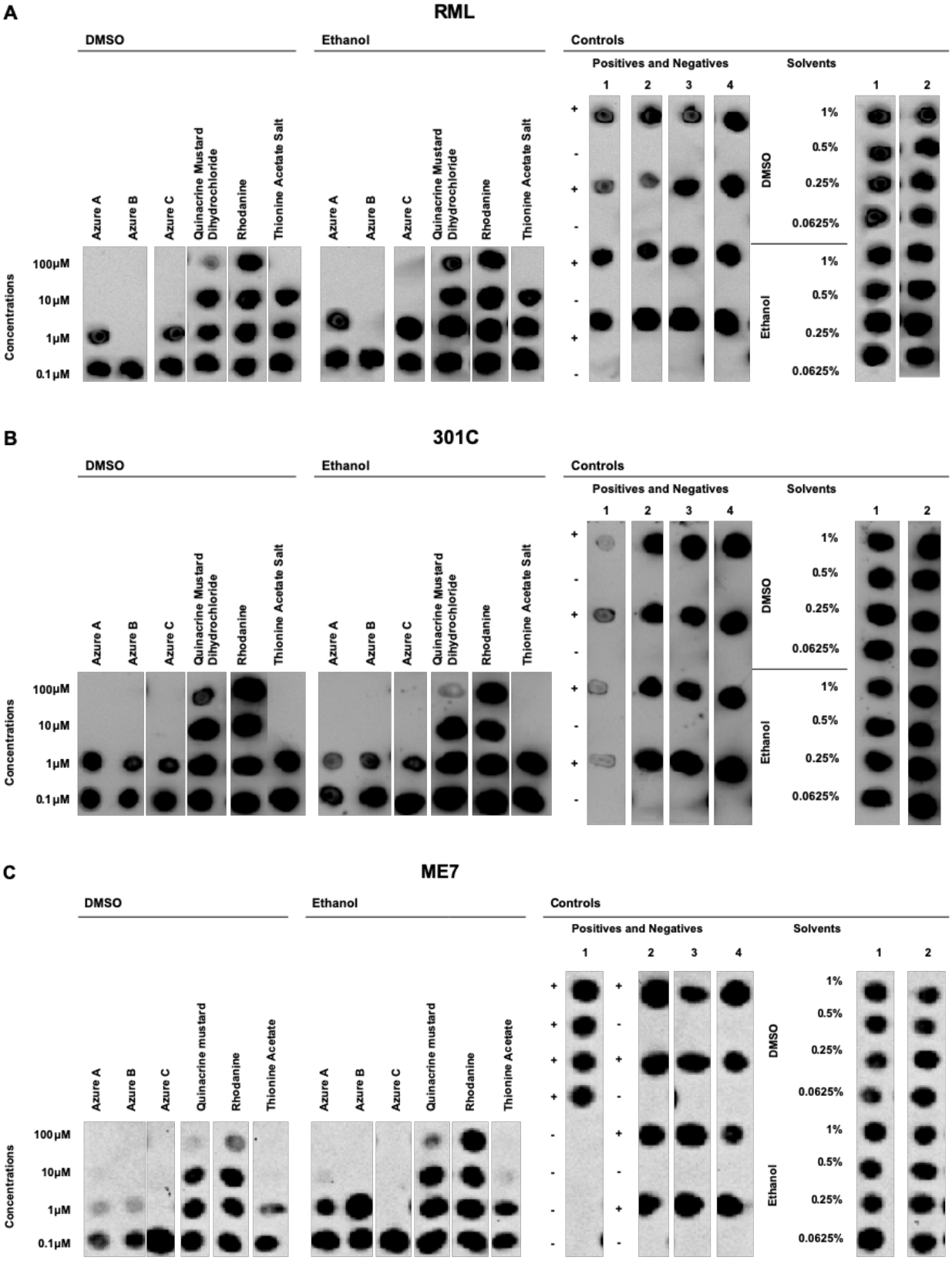

A
RML
DMSO
Ethanol
Controls
Positives and Negatives
Solvents
Azure A
Azure B
Azure C
Quinacrine Mustard Dihydrochloride
Rhodanine
Thionine Acetate Salt
Concentrations
100µM
10µM
1µM
0.1µM
1
2
3
4
DMSO
Ethanol
1%
0.5%
0.25%
0.0625%
B
301C
C
ME7
Quinacrine mustard
Thionine Acetate

Figure 13. Evaluation of known anti-amyloid small molecules in 96wp-PMCA (mouse prion strains). Six known anti-amyloid compounds were tested at four different concentrations in DMSO and EtOH for their activity against RML (A), 301C (B), and ME7 (C) *in vitro* replication. Controls for solvents and positive samples and negative samples without compounds were placed on the plate to control the performance of the assay. Dot blots were modified for labeling. Figure is representative from three independent assays.

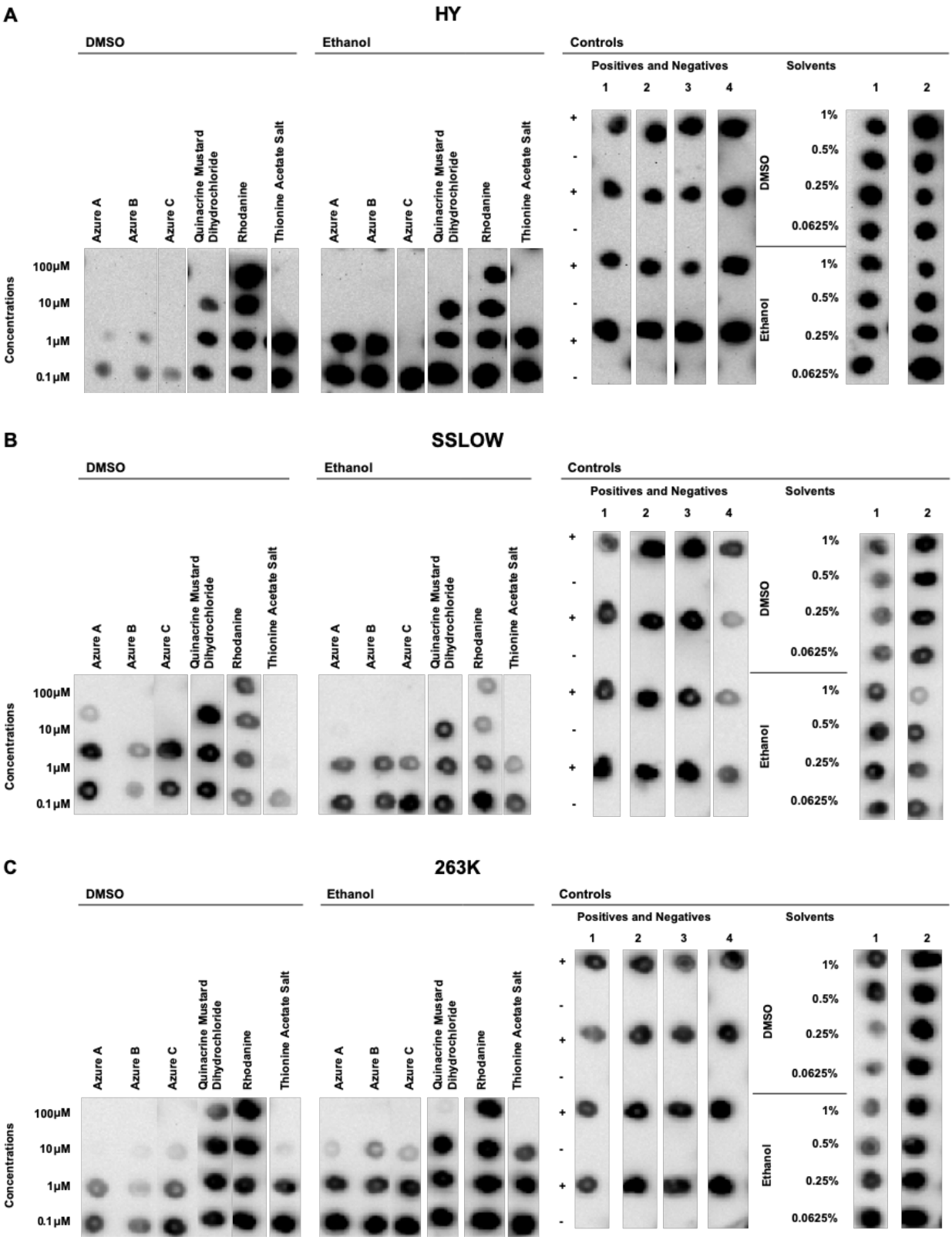

A
HY
DMSO
Ethanol
Controls
Positives and Negatives
Solvents
Azure A
Azure B
Azure C
Quinacrine Mustard Dihydrochloride
Rhodanine
Thionine Acetate Salt
Concentrations
100µM
10µM
1µM
0.1µM
1
2
3
4
DMSO
Ethanol
1%
0.5%
0.25%
0.0625%
B
SSLOW
C
263K

Figure 14. Evaluation of known anti-amyloid compounds in 96wp-PMCA (hamster prion strains). Six known anti-amyloid compounds were tested at four different concentrations in DMSO and EtOH for their activity against HY (A), SSLOW (B), and 263K (C) *in vitro* replication. Controls for solvents and positive samples and negative samples without compounds were placed on the plates to control the performance of the assay. Dot blots were modified for labeling. Figure is representative from three independent assays.

DISCUSSION AND CONCLUSION

The Protein Misfolding Cyclic Amplification (PMCA) technology was an appropriate option to be used for this project due to (i) its ability to replicate the conformational properties of infectious prions while maintaining all their biologically relevant features (including strain specific properties, (61)), (ii) each assay can be completed in a relatively short time, (iii) it represents a low cost technology, suitable for screening large number of samples. Based on the other applications of PMCA, I believed that the technique could be optimized for high-throughput identification and evaluation of small molecules with presumed anti-prion activity.

High-throughput screening (HTS) allows for a large number of samples to be tested against a specific target. This is performed by the utilization of automatization and reduction of assay components allowing for the process to occur effectively, efficiently, reproducibly and at minimum cost (89). A selected number of modifications would need to occur in order to achieve the development of a high-throughput PMCA (HT-PMCA) platform. In my project, I did not achieve the establishment of a HT-PMCA platform since, at its current status, my optimized platform allows the screening of compounds at a medium throughput range. However, I believe that the establishment of a 96-well plate PMCA (96wp-PMCA) assay represents an important step forward in that direction. Modifications of PMCA that I aimed to achieve with the 96wp-PMCA included the decrease amount of time and volume that is required to complete the assay. In comparison to conventional PMCA, I also decreased the number of teflon beads required, lowered the volume of material to be utilized, substantially increased the number of samples to be tested per PMCA assay, and decreased the length of the overall process (Figures 2-5 and Table 1). All these manipulations were effective in reducing the overall cost associated with this technique.

The first step on this optimization path was to determine the minimum volume of samples that would be required to allow for proper analysis. Importantly, the selection of such volume should fulfill the criteria of being reproducible across the whole 96 well plate in independent assays. In order to establish this volume, it was crucial to determine the volume that would be required to evaluate the samples with dot blot. I directly placed three different volumes of 10% w/v brain homogenate prepared from terminally ill mice infected with the RML prion strain onto a nitrocellulose membrane (Figure 2). From this experiment, I determined the volume of the sample required for dot blot analysis to be 5 µL. In parallel, I also determined that the PMCA reaction can be performed in a volume as low as 50 µL. The latter assay volume was determined to be the minimum possible since it allows for 2 teflon beads to be completely submerged (Teflon beads are known enhancers of PMCA while buffering reproducibility across different assays (90–92)). The beads are able to assist in the breaking of the PrP^{Sc} and provide additional surface area for the conversion of PrP^{C} to PrP^{Sc} to occur. A significant amount of time in the conventional PMCA assay was attributed to the incubation period of the primary antibody. Therefore, it was my goal to decrease the time dedicated to this critical step (Figure 3). Since I saw comparable results when comparing the 16 hrs and 1 hr incubation time, I was able to determine that 1 hour was sufficient for my purposes.

I can see that PMCA is unable to occur effectively when EDTA is not supplemented into the brain homogenate (Figure 4). Although the mechanism is unclear, the presence of cations has been hypothesized to prevent or delay the replication process (84, 85). Since PMCA is a process that focuses on protein-protein interactions, I hypothesize that the cations in solution may be interacting with either PrP^{Sc} or PrP^{C} preventing the desired interaction to occur. Consequently, the usage of 1 mM EDTA is shown to render these cations unable to hinder the PMCA process. The purpose of the

results shown in Figure 5 focused on finding the lowest dilutions where amplification can be seen uniformly across the plate. I was able to see uniform amplification for the RML strain at 1 x 10^{-5} % w/v; however, for other strains (not shown) uniform amplification was only seen up to a dilution of 1 x 10^{-4} % w/v. In that sense this dilution of infectious prions was used to supplement the 96wp-PMCA reactions in future assays.

With the transition from using PCR strip tubes to a 96-well plate, it was necessary to optimize 96wp-PMCA for more than one prion strain. Optimizing the platform for a selection of strains allows for the accommodation of different species and distinct characteristics associated with each strain. Importantly, and due to the high sensitivity of the PMCA assay (93), I had to verify the validity of the technique through the presence of negative control samples (to assess for possible inter-well cross-contaminations). The first step included testing the performance of six different rodent prion strains in my assay, considering all above-mentioned optimized conditions and requirements. In summary, I successfully achieved optimal 96wp-PMCA assays for mouse (Figure 6) and Syrian hamster (Figure 7) prions. Through the random placement of negative controls for each of the prion strains tested, I am able to conclude that uniform amplification of the samples occurred regardless of their placement location on the plate. Biochemically, prion strains can be distinguished by western blot due to their differential PK resistant core (different lengths) or their glycosylation profiles (47). One of the limitations of using dot blots for analysis of PMCA-derived samples from multiple strains is the incapability to distinguish them. To account for this restriction, each assay only tested a single prion strain and the signal was assessed in an all-or-none fashion. However, to verify that the PMCA did not alter the strain properties of each selected agent, I ran selected samples by western blots (not shown).

The next step in my proposed project was to validate the 96wp-PMCA assay for the screening of small molecules harboring anti-prion activity. As regular practice, compounds from libraries are dissolved in different solvents, DMSO and EtOH being the most widely used. These solvents were evaluated independently in the 96wp-PMCA to determine if they would influence the prion replication process. Inhibition was seen at 8% v/v EtOH in both mouse and hamster prion strains. Additionally, I also observed inhibition at 4% EtOH with the HY prions (Syrian hamster strain). On the other hand, I did not see any inhibition with DMSO at any of the concentrations tested for the mouse or hamster strains. Despite the ability for these solvents to inhibit the *in vitro* conversion of some of the tested prion strains, I was not majorly concerned since I dissolved all molecules at 1% v/v of each or both solvents. Since the solvent concentration chosen does not show hinderance of *in vitro* conversion, any inhibition observed in the presence of small molecules can be attributed to the molecule itself.

The majority of the anti-prion molecules tested in this study (Figures 10 & 11) were selected on their ability to inhibit RML in neuroblastoma cell lines at nanomolar concentrations (49). Thioflavin T was chosen as a molecule to test due its ability to nonselective intercalate into the backbone of several classes of amyloids (94, 95). Since my paradigm consists of an *in vitro* platform for the conversion of PrP^{C} to PrP^{Sc}, I hypothesize that effective molecules will influence protein protein interactions through (i) binding to the PrP^{C} and preventing its conversion to PrP^{Sc}, (ii) binding to PrP^{Sc} and prevent the recruitment of PrP^{C}, (iii) targeting the particular glycosylation preferences of each prion strain, or (iv) binding to still unknown co-factors present in the brain homogenate that could alter the conversion of each specific prion strain.

Only three of the anti-prion molecules were effective against one or more mouse prion strains (Figure 10). Specifically, all three strains were inhibited by 10 μM tannic acid; however, strain specific inhibition was seen for Congo red and thioflavin T. Although RML and ME7 are both derived from scrapie (47), they did it from different sources of this infectious agent. Consequently, both mouse-adapted prion strains display different glycosylation profiles and generate different lesion patterns in brains of experimental subjects. This information strongly supports the notion of both being different "conformational" strains, and that may be a leading reason as to why Congo red and thioflavin T inhibited RML but not ME7. Congo Red did not show selective strain inhibition for the hamster prion strains tested in this study, even at the highest concentration tested (100 μM). In the same line, I observed that curcumin selectively inhibited the replication of HY and 263K prions, but not SSLOW (Figure 11).

Tannic acid is considered to be a polyphenol inhibitor of amyloid aggregation and found naturally in tea, red wine, and nuts. Based on previous studies performed to studied its inhibition against prions (49), its mechanism is believed to be through direct interactions with PrP. Considering inhibition was observed in all six strains but required a high concentration for inhibition of HY when dissolved in DMSO, I predict that tannic acid's mechanism may be altered due to the specific biochemical characteristics of HY. Congo red and Thioflavin T are both used as dyes in histological studies in order to visualize amyloid deposits (96). These dyes are believed to interact with the β-pleated sheet found in the secondary structure of amyloid aggregates (97). The usage of these dyes as inhibitors would suggest that through inserts into the deposits, they prevent the recruitment of PrP^{C} by PrP^{Sc}. The lack of inhibition seen with ME7 for both of these compounds would indicate that the unique conformation associated with this strain

protects its β-pleated sheet and does not allow for the molecules to bind. As for the other five strains, these dyes may not only be interrupting the conversion process by (1) blocking the interaction between PrP^{C} and PrP^{Sc} but also (2) obstructing the ability for PrP^{Sc} to misfold properly thus further disrupting the assembly of the aggregate.

Quinacrine is considered to be an anti-malaria treatment (98). Concerning quinacrine as a treatment option against prions, studies have been conducted in cell models, animal models and in human clinical trials. In cell model studies tested against scrapie, it was reported that quinacrine reduces the growth of abnormal prion protein deposits (49); however, when tested against chronic wasting disease, it aided in the accumulation of PrP^{Sc} (99). When administered in mice models against a mouse adapted BSE strain, a decrease in PrP^{Sc} was described (100) and had no effect in humans with sporadic CJD patients (101). Since numerous of studies indicted the decrease in PrP^{Sc} levels, I believe that the mechanism of quinacrine would be at the protein-protein level which makes my technique of 96wp-PMCA a viable platform to test this theory. Considering no inhibition was observed against any of the strains, possible explanations for this could be (1) quinacrine does not inhibit by binding to either forms of the prion protein, (2) the usage of sonication breaks any interaction that may be form between quinacrine and the prion protein, or (3) similar to chronic wasting disease, quinacrine helps in the creation of PrP^{Sc} of the strains tested.

Similarities can be seen between prion diseases and other neurogenerative diseases, such as Alzheimer's and Parkinson's disease. These diseases are associated with the misfolding of proteins and histological dyes such as thioflavin to bind to misfolded protein aggregates despite the protein composition of these aggregates (95). Based on this knowledge, I postulated that anti-amyloid molecules may be effective in inhibiting

prions as well. The anti-amyloid molecules (Figures 13 & 14) tested in this project have been shown to inhibit the aggregation of protein linked to Alzheimer's and Parkinson's disease. Azure A, azure B, azure C, and thionin acetate all belong to a class of drugs called phenothiazines (76) and have all been shown to inhibit Aβ aggregation and tau filament formation. Rhodanine inhibits the aggregation of tau (102) and quinacrine mustard is considered to be a structural relative of quinacrine which in turn may have a different inhibitory effect. When comparing the anti-amyloid molecules' effectiveness against the mouse prion strains (Figure 13) in comparison to the anti-prion molecules, I was able to see that two of the molecules inhibited prion replication at a concentration as low as 1 μM. This contrasts with the lowest concentration found for the anti-prion molecules, from which the most effectives worked at a minimum concentration of 10 μM. The difference in the chemical structure of azure C in comparison to azure A and azure B can be the reasoning behind the lower concentration of inhibition seen when tested against HY.

Inhibition was seen with all four phenothiazines. When azure A and azure B were used in mouse models against AD, it was shown to stimulate protein degradation (103). Assuming these molecules are not selective towards amyloid-β and various inhibition concentrations seen between these two molecules along with azure C for the six strains, the inhibitions seen can be attributed to the success of each molecule to promote PrP degradation. Due to the utilization of PMCA to evaluate these molecules, I am unable to determine which form of the prion protein is targeted. Azure A, azure B, and thionin acetate are used as a dye and their mechanism of binding is through intercalation (104). Since thioflavin T follows a similar mode of action, I believe that these three molecules could be behaving very similar to thioflavin T. Comparing the structures of these

compounds to thioflavin T, they all have three cyclic structures. Further studies would be required; however, these structures could aid in their intercalation into the misfolded protein.

It is important to note that quinacrine is typically accepted as a good anti-prion drug compared to its structural relatives (75, 100); however, for the hamster strains, I am able to see a better performance for quinacrine mustard instead of the parental structure. The difference in inhibition could be as a result of the presence of the two chloride functional groups attached to the quinacrine mustard dihydrochloride. Because inhibition with quinacrine mustard was only seen with hamster prion strains binding to cofactors necessary for prion misfolding which are present in the hamster brain extract but not in the mouse counterpart may assist in the species-specific inhibitory effect of this molecule. Further *in silico* studies may assist in clarifying the relationship between small molecule composition and their interaction with prions in a strain-specific manner.

It is important to mention that due to the great sensitivity and strength of the PMCA technology (93), it is not surprising that some molecules worked at lower concentrations compared to the ones described using other systems. In that sense, the novel anti-prion activity of some molecules from the “anti-amyloid” group look promising for future studies. Unfortunately, due to the constraint of the technique utilized, the molecules were only tested for their ability to inhibit protein-protein interactions. This could also explain the lack of inhibition seen for some of the previously described anti-prion molecules working at nanomolar concentrations in cell-based systems (49). Despite six of all the molecules showing no inhibition at the concentration tested, strain-specific inhibition can be seen for five of the molecules in hamster prions and four of the molecules in mouse prion strains. When comparing the molecules between both mice and hamsters, further strain specific inhibition can be seen. Based on these results, my original hypothesis is

supported and confirm the need for strain-specific treatment options. I believe that the current PMCA format can easily be applied towards testing a library of compounds showing to inhibit other amyloids, as well as larger libraries. The optimization of PMCA for high throughput screening will provide for another route to find treatment options for prions along with being utilized in the diagnostic process of prion disease. Another benefit and application of the HT-PMCA technique is the ability to apply it towards the study of proteins involved in other neurodegenerative diseases, such as, Alzheimer's and Parkinson's.

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