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Therapeutics for Prion Disease: Approaches with Lysine-based Compounds

A Thesis Presented for the Master of Science Degree

The University of Tennessee at Martin

Karen Sue Jackson

May 2011

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Abstract

Prion diseases are lethal neurodegenerative disorders caused by infectious pathogens termed prions. They are composed of PrP^{Sc}, a misfolded isoform of the cellular prion protein. Prion diseases include scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease in deer and elk, and Kuru and Creutzfeldt-Jakob disease in humans. As exemplified by cross-species transmission of BSE to humans, prion diseases pose a risk for human public health. Presently, no cure or therapy is available for these devastating diseases.

In previous research to develop anti-prion agents, polymers of lysine (poly-L-lysine; PLK) exhibited inhibitory effects on prion propagation in both cell-based and animal models of disease. Since PLK is known to be toxic to both cellular and animal models, an attempt was made to decrease toxicity by attaching polyethylene glycol (PEG) to PLK. Comparative studies were performed on anti-prion activity and cytotoxicity using PLK and its modified derivative (PEG-PLK). Cytotoxicity studies, measured by a thiazolyl blue tetrazolium bromide (MTT) assay, in prion-infected neuroblastoma cells incubated with PLK and PEG-PLK, revealed that PEG-modification decreased cytotoxicity associated with PLK. By comparing PrP^{Sc} levels, PEGylation was observed to decrease the efficacy of PLK to inhibit prion propagation. However, PEG-PLK maintained the ability to completely eliminate PrP^{Sc} at higher concentrations (10 μM), as determined by

western blotting and densitometry. Another experiment was designed to study the cytotoxicity and anti-prion activity of poly-D-lysine (PDK), the stereoiosomer of PLK. In measurements of cytotoxicity in prion-infected cells, PDK was more toxic than PLK. The cells incubated with concentrations less than 50 µg/mL PLK survived, while they did not if incubated with concentrations greater than 10µg/mL PDK. The lethal concentrations (LC50) of PDK and PLK were 0.13µg/mL and 31µg/mL, respectively. However, PDK at 0.25 µg/mL showed anti-prion activity, while no anti-prion activity was observed with PLK below 2 µg/mL. The inhibitory concentration (IC)50 of PDK and PLK were 3 µg/mL and 1.25 µg/mL, respectively. Anti-prion activity of PDK was still below the cytotoxicity limits set by the MTT assay.

PEG-PLK had decreased cytotoxicity in vitro compared to PLK. PEG-PLK had decreased anti-prion activity when compared to PLK, but still reduced prion levels to below detectable limits. PDK had more anti-prion activity than PLK, but was also more cytotoxic than PLK. However, PDK reduced prion levels to below detectable limits at concentrations below the LC_{50} determined by MTT assay.

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List of Abbreviations

µg Microgram

µL Microliter

µM Micromolar

A alanine

APS ammonium persulfate

ATCC American tissue culture collection

BCA bicinchoninic acid

BSA bovine serum albumin

BSE bovine spongiform encephalopathy

CJD Creutzfeldt-Jakob disease

CWD chronic wasting disease

DMEM Dulbecco's modified Eagle's medium

Doc sodium deoxycholate

DPBS Dulbecco's phosphate buffer saline

FBS fetal bovine serum

GLP good laboratory practices

GPI glycosyl phosphatidyl inositol

H histidine

HCl hydrochloride

Hum-P humanized anti-PrP antibody clone P

IC_{50} Concentration at which 50% of the PrP^{Sc} was inhibited

KCl potassium chloride

LC_{50} Concentration at which 50% of the cells were killed

MBM meat and bone meal

MeOH methanol

mL Milliliter

MTT thiazolyl blue tetrazolium bromide

NaCl sodium chloride

NP 40 nonylphenylpolyethylene glycol, Nonidet P-40

PAMAM polyamidoamine

PDK poly-D-lysine

PEG polyethylene glycol

PEI polyethyleneimine

Pen penicillin

PK protease K

PLK poly-L-lysine

PMSF phenylmethanesulfonyl fluoride

PrP prion protein

Q glutamine

R arginine

RML Rocky Mountain Laboratory

SAF scrapie associated fibrils

ScN2a N2a cells infected with scrapie

SDS sodium dodecyl sulfate

SLB sample loading buffer

SSCA standard scrapie cell assay

Strep streptomycin

TBST Tris buffer saline with Tween-20

TEMED tetramethyl-ethylenediamine

TG Tris/ glycine

TGS Tris/ glycine/ SDS

USDA United States Department of Agriculture

V valine

Chapter 1 Literature Review

Introduction

Prion diseases are fatal neurodegenerative disorders caused by infectious pathogens termed prions. They are composed of PrP^{Sc}, a misfolded isoform of the normal cellular prion protein (PrP^{C}). Prion diseases include scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in deer and elk, and Kuru and Creutzfeldt-Jakob disease (CJD) in humans. As exemplified by cross-species transmission of BSE to humans causing variant CJD in Europe, prion diseases pose a risk for human public health. Presently, no cure or therapy is available for these devastating diseases.

The History of Prion Disease

Scrapie was first documented in the 18th century (1). Sheep were being inbred to improve wool quality for the English woolen market. As the inbreeding increased, so did the incidence of scrapie (1). As the woolen market decreased near the end of the 19th century, the need for high quality wool decreased. Sheep breeding began to focus on the meat market, so the incidence of inbreeding decreased, which led to a decrease in scrapie. In the mid-19th century, a scientific study was conducted by veterinarians from France, England, and Germany to determine the infectious agent in scrapie. Bensoit and his colleagues characterized neuronal vacuolation in the disease, but failed to transmit it to a healthy sheep from inoculation of brains and

blood transfusions from infected sheep (1). In 1936, scientists discovered that scrapie had a long incubation time, of one to two years.

Cuillé and Chelle (1) subcutaneously, intracranially, and peripherally inoculated sheep with scrapie infected brain and spinal cord tissue and found that incubation times varied with the method of inoculation. Intracranially inoculated animals had the shortest incubation time until disease onset, while the peripheral inoculation method resulted in the longest incubation time (1). In an independent English study, scrapie was introduced into many flocks during vaccination of young sheep to prevent louping ill (2)[A]. The louping ill vaccine was made from formalized infected sheep brain from a sheep exposed to louping ill. Later, the sheep used to generate the vaccine was found to have scrapie (1). This confirmed the findings of Cuillé and Chelle. In 1965, Carleton Gajdusek experimentally transmitted the human prion disease Kuru from humans to chimpanzees following a two year incubation period. (3) Later, scrapie was experimentally transmitted from sheep to cattle, (4) and BSE was experimentally transmitted from cattle to sheep and humans (5). CWD has been experimentally transmitted from deer to cattle (6). The ability of prion disease to transmit across the species barrier confirms the transmissible nature of the disease.

A BSE outbreak drew interest during the 1990's. The first case of BSE was diagnosed in 1986 (7). In 1993, the incidence of BSE peaked. Wilesmith et al. (8)

[A] Louping ill is a tick borne, fatal, zoonotic disease that usually affects young sheep that have lost the maternal antibodies.

discovered several risk factors for the spread of prion disease while studying BSE data and the its incubation time in cattle. In his research, Wilesmith looked at risk factors such as diet, vaccines, hormones, and insecticide treatments. The most common risk factor was a protein supplement, meat and bone meal (MBM), found in weaning rations for calves. This was consistent for both dairy and beef breeds, but dairy calves had a higher incidence of BSE than beef calves. Since dairy calves are fed via feedlot and beef calves are fed via pasture, this was consistent with the hypothesis that MBM is linked to BSE. As a result, the process for production of MBM was reviewed. In the 1970's, some changes had been made to the process for producing MBM. MBM is a product of the offal of slaughtered animals. The MBM in the United Kingdom contained both sheep and cattle offal. Scrapie in sheep had a high incidence rate in the United Kingdom (two animals per 1,000 are infected) (7). Prior to the 1970's, rendering plants produced both tallow and MBM from the offal. During production, the offal was ground up and heated with steam. Then it was milled and the tallow was extracted. This process required a high energy input that became cost prohibitive with the increase in fuel prices in the 1970's. At the same time, the price of tallow decreased compared to the price of MBM, which made the process more expensive (7). Because it was not economical, the process of removing tallow was discontinued.

Scientists then became concerned with the physical properties of prions. PrP is attached to cellular lipid membranes with a glycosyl phosphatidyl inositol (GPI)

anchor (7). Prions are resistant to heat and most harsh denaturing substances used to inactivate lipid solvents. When tallow removal stopped, the infected lipids were still present in the MBM allowing prions to infect animals that consumed MBM. The incidence of BSE in the United Kingdom gradually increased from 1987 to 1990 (Figure 1). There was an exponential increase in the incidence of the disease from 1990 to 1993. After 1993, the incidence of BSE in cattle gradually decreased. In 1988, the United Kingdom banned re-feeding ruminant MBM to ruminants which led to the decrease of BSE after 1993.

What is a Prion?

A prion is widely believed to be a misfolded isoform of the normal, ubiquitously expressed cellular protein, PrP^{C}. It is abundant in neuronal cells of the brain and spinal cord. PrP^{C} attaches to cellular lipid membranes with a GPI anchor (7). In PrP^{Sc}, some of the α-helices of PrP^{C} are transformed into β-sheets (Figure 2) (8). Although both PrP^{C} and PrP^{Sc} share the same primary amino acid sequence, PrP^{Sc} has β-pleated sheets refolded from a portion of α-helix random coil structure of PrP^{C} (Figure 2) (9). Because of the decrease in number of α-helices and increase in number of β-sheets, PrP^{Sc} is less soluble and more resistant to proteases (10). The decreased solubility and increased resistance to proteases results from the hydrophobic nature of PrP^{Sc}. The increased β-sheet content exposes more negative hydrogen ions. In normal PrP^{C}, the α-helixes protect the hydrogen ions by surrounding them with amino acids. These changes cause aggregates of PrP^{Sc} that

accumulate in the neurons of affected brain areas (11). This process is considered to lead to synapse degeneration and finally neuronal death (12). X-ray crystallography and nuclear magnetic resonance studies revealed that PrP^C had a largely unstructured N-terminal region and a stable C-terminal globular domain. It showed three α-helices and two short anti-parallel β-sheets (Figure 2) (13). According to Fourier-transformed infrared spectroscopy, PrP^{Sc} contained 30% α-helix and 43% β-sheet. This contrasts with normal PrP^C that contains 42% α-helix and 3% β-sheet (13). When PrP^C converts to PrP^{Sc}, the β-sheet content increases significantly while the α-helix content decreases. This is why an increased number of β-sheets is indicative of prion disease formation. The β-sheets primarily occur in the N-terminus region (13).

Clinical Symptoms of Prion Disease

Clinical symptoms of scrapie vary among individual animals, breeds, and species of sheep and goats. Early clinical signs of scrapie can be observed through slight temperamental changes in the animal. As the disease progresses, the infected animal will start to tremor and lose coordination (14). Diseased sheep and goats continue to weaken and lose weight, despite having voracious appetites. Sheep tend to lose hair, due to the compulsive behavior of scratching fenceposts and trees, but goats tend to nibble their own hair (14). The most accurate diagnosis of scrapie is the post-mortem test. Brain tissue can be observed for signs of scrapie in the animal. Samples are taken from the thalamus, midbrain, pons, medulla, cerebellum, anterior spinal cord, hippocampus and cerebrum and stained with hematoxylin and eosine for

microscopic observation. The samples are microscopically observed for lesions that have caused neuronal degeneration and spongiosis. Any observed lesions are studied for neuronal shrinkage. A positive scrapie associated fibrils (SAF) test is now a requirement for a diagnosis to be considered scrapie (15). SAFs are only found in animals and humans that test positive for PrP^{Sc}; they are not found in normal brains and spinal cord tissue. SAFs were first noticed when tissue samples were treated with detergent and viewed through electron microscopy with a negative stain (16). SAF testing uses immunocytochemical staining with antisera raised against specific scrapie strains. The antisera indicate the amyloid plaques that are associated with SAFs and their corresponding PrP^{Sc} strains (16).

In many cases, western blotting is used to determine PrP^{Sc} levels in the laboratory. Protease K (PK) is used to digest the samples because PrP^{Sc} is resistant to PK digestion. Western blotting is then used to separate out the prion proteins so that they can be easily detected. When scrapie is detected via western blotting, three bands appear near 27-30 kDa and below 25 kDa. Without these three bands, there is no indication that PrP^{Sc} is present in the sample. The three bands indicate the three scrapie associated fibrils, which only show on tests from scrapie infected animals after PK digestion.

Genetic and Environmental Factors Involved in Transmission of Prion Disease

There are several factors involved in the transmission of prion disease. One factor is the genetics of the animal. Genetic testing can be used to determine if an

animal is susceptible to scrapie. The genetics of goat scrapie susceptibility are relatively unknown, but there have been correlations of isoleucine (I) with codon 142, when I_{142} is found homozygously and heterozygously (14). Goats with the I_{142} polymorphism are more susceptible to scrapie (14). There are several known polymorphisms in sheep that increase susceptiblity to scrapie. These polymorphisms most commonly occur at (Alanine$_{136}$ (A)/ Valine$_{136}$ (V)), (Arginine$_{154}$ (R)/ Histidine$_{154}$(H)), and (Arginine$_{171}$ (R)/ Glutamine$_{171}$ (Q)) (17). The alleles associated with the codons are $V_{136}R_{154}Q_{171}$, $A_{136}R_{154}Q_{171}$, $A_{136}H_{154}Q_{171}$, $A_{136}R_{154}H_{171}$, and $A_{136}R_{154}R_{171}$. $V_{136}R_{154}Q_{154}$ is the most scrapie susceptible allele variant. When V_{136} was present in both Cheviot and Swaledale sheep, scrapie susceptibility increased (17). In Cheviots, the homozygous AA_{136} (AA_{136}) / homozygous QQ_{171} (QQ_{171}) variant protected against scrapie when it was introduced into the flock. The next susceptible variant was $A_{136}R_{154}Q_{171}$ and $A_{136}H_{154}Q_{171}$ in the Suffolk breed. Homozygous RR_{171} (RR_{171}) or heterozygous RH_{171} (RH_{171}) protected the Suffolk against scrapie when it was introduced into the flock. When QQ_{171} was present in the Suffolk breed, the susceptibility to scrapie increased (17). The susceptibility of the breed to scrapie was detected in sheep by testing the polymorphisms of the PrP alleles. The least susceptible alleles were $A_{136}R_{154}H_{171}$ and $A_{136}R_{154}R_{171}$. When scrapie was introduced, no sheep with the alleles $A_{136}R_{154}H_{171}$ and $A_{136}R_{154}R_{171}$ contracted the disease.

Various polymorphic amino acid sequences in the PrP gene either increase or decrease the susceptibility to prion disease. Although the molecular mechanism for this phenomena is not clearly understood, it has been speculated that substitution of amino acids to charged residues may contribute to formation of PrP^C with certain conformations, which can direct the efficacy of PrP^C conversion to PrP^{Sc}.

In addition to genetic factors, transmission of prion disease in animals can be influenced by environmental factors. Sheep and goats can be exposed to scrapie from other animals in the herd. This is considered a horizontal transmission. Scrapie and CWD can be transmitted within a herd or flock through milk (18), saliva (19), urine (19), and feces (20). Madison et al. (21) studied the exposure of sheep to prions in the sheep environment. Sheep flocks were divided into two categories, scrapie positive and scrapie free sheep. Swab samples were taken from plastic posts, metal water and feed troughs, gates, fences, and a wooden post after the sheep were removed from the area. Samples were collected for one week and then analyzed using protein misfolding cyclic amplification (PMCA) to amplify the PrP^{Sc} in the samples. PMCA uses two steps to analyze the samples (22). The first cycle has both PrP^C and PrP^{Sc} which were incubated to induce misfolding of PrP^C to PrP^{Sc}. The aggregates of PrP^{Sc} were then broken down through sonication during the second cycle to multiply the number of PrP^{Sc} seeds. The increased amount of PrP^{Sc} can then be detected with western blotting analysis. In the pasture with the scrapie positive flock, PrP^{Sc} was found on all surfaces, both indoor and outdoor, except for the metal

water trough and the gate. There was no PrP^{Sc} found on any of the surfaces contacted by the scrapie free flock. Therefore, this study (22) showed that sheep and goats could potentially contract the disease if animals lick surfaces containing PrP^{Sc}.

Animals can also be exposed to prions through the soil. According to both Brown (23) and Seidel et al. (24), scrapie prions retain their infectivity in the soil for several years. Nagaoka et al. (25) showed that soils should be considered contaminated for up to three months after scrapie had been introduced. After three months, the contamination is so small that problems should not occur in animals grazing on the land (24). However, the contamination period is further prolonged when the prion binds to minerals in soils (26) Yang et al. (27) studied conformational changes in PrP when it binds with copper. In particular, Yang et al. investigated factors that influenced copper-binding and the structural changes of the polymorphism on PrP. Two polymorphic variants of $A_{136}R_{154}Q_{171}$, M112T, of the sheep allele M112T were studied. Sheep with polymorphism M112T alleles are more resistant to scrapie infection than sheep having the wild-type M112T allele. The wild-type M112T protein has improved thermostable properties and is more susceptible to copper treatment than the polymorphic variant M112T. The wild-type M112T shows a greater increase in β-sheet content than the polymorphic variant M112T when treated with copper.

The amount of copper in the soil could potentially increase length of time the prions remain infectious in the soil. Mitteregger et. al. (28) found that a diet with a

low level of copper hastened prion disease in mice, whereas, a diet with significantly higher levels of copper (compared to the control group) delayed the onset of prion disease. According to Brown et al. (29), copper binds to the prion N-terminal sequence in vivo. The role of the prion-bound copper ions is currently unclear. Brown et al. (29) suggests that PrP^C might act as a "shuttle" for copper ions destined to bind to enzymes that prevent oxidative damage, although more active roles are being considered as well. Miura et al. (30) suggest that the copper ions might induce α-helical motif in the N-terminus of PrP to act as a template for formation of the α-helices in the rest of PrP. This research shows that there is a potential for prions to spread through the soil, if they are low in copper. At a minimum, low copper content could allow prions to harbor in the soil for much longer than they normally would.

Control of Prion Disease

Presently, there are no available treatments for prion disease. Prion disease is controlled with preventive measures. Genetic testing is used to detect the susceptibility of an animal to prion disease. The USDA presently uses the presence of arginine (R) at Codon 171 of the PrP^C protein as an important determinant of scrapie susceptibility in sheep. (31) According to the USDA, animals that have R at Codon 171 have a decreased incidence of scrapie. To determine this, the USDA studied production traits of five sheep breeds (Columbia, Rambouillet, Hampshire, Suffolk, Targhee) and one cross bred group with the R_{171} allele. Three groups of animals were genotyped: 585 ewes, 947 rams, and 901 lambs. All five breeds and

the commercial western white-faced ewes showed the R_{171} allele. Columbia, Rambouillet, and Hampshire ewes were not affected on either multiple births or total weight for lambs weaned in this study (31). The Suffolk ewes that had the non-R/non-R allele produced more lambs than the heterozygous non-R/R ewes. Homozygous non-R/non-R Suffolk ewes had a heavier total weight of lambs, but less weight for individual lambs than heterozygous non-R/R. In contrast, the commercial flock ewes had more multiple lambs with homozygous R/R, but there were no noticeable differences in the total weight of lambs weaned.

The above research explains why culling a genetically susceptible animal is often impossible. Commercial ewe flocks contain ewes of mixed ancestry. The ewes genotyped homozygous R/R had more multiple lambs, yet there were no discernible total weight differences. In commercial flocks, ewes with the desirable homozygous R/R allele will be selected. In contrast, Suffolk ewes that have the homozygous non-R/non-R gene are selected due to the fact that they produce more multiples. The heterozygous non-R/R ewes had higher individual weights, but lower total weights of lambs. This is likely due to the decrease in multiples of the heterozygous non-R/R gene. Typically sheep that do not produce twins or wean at a minimum of 50% of their body weight are culled from a flock. According to these results (31), by increasing the overall profit margin, the producer would eventually eradicate the desirable heterozygous non-R/R gene out of the Suffolk herd, inadvertently increasing overall flock susceptibility to scrapie.

Potential Treatments for Prion Disease

Possible treatments for prion disease include glimepiride (12) and polyamines (32). Glimepiride is already approved to treat diabetes in humans. When used to treat prion disease, glimepiride releases PrP^{C} from the surface of prion-infected neuronal cells (12). The cell surface is a possible site for PrP^{C} conversion to PrP^{Sc}. While the glimepiride was being used, the cortical and hippocampal neurons were protected from the neurotoxicity induced by PrP (82-146), a synthetic peptide containing amino acids 82 to 146 of human PrP (12). Glimepiride also reduced the amount of activated cytoplasmic phospholipase A2, which began synapse degeneration and neuronal death. Prostaglandin E2, which confirms cytoplasmic phospholipase A2 activation, decreased (12).

Complex polyamines constitute the sole class of compounds that exhibit the ability to eliminate pre-existing PrP^{Sc} molecules from infected cells (32). The branched polyamine potency, such as cationic dendrimers, intensifies with the density of positive charges on the surface of polyamine molecules. These dendrimers appear to group with PrP^{Sc} molecules in the lysosomes, where the acidic environment aids dendrimer-mediated PrP^{Sc} disaggregation.

Despite the ability of branched polyamine compounds to interact with specific epitopes of each protein, the toxicity associated with them often interferes with the successful application of the compounds to treat prion disease. To reduce toxicity while keeping the ability of the compounds to treat PrP^{Sc}, quaternized amines have

been added. Polyethyleneimine (PEI) and polyamidoamine(PAMAM) dendrimers improved the range of nontoxic concentrations of the compounds while maintaining a reduced level of anti-prion activity (32). Furthermore, quaternized PEI was able to degrade PrP^{Sc} in acidic conditions and reduce the in vitro PrP^{Sc} progagation facilitated by conversion of the normal PrP^{C} isoform to its misfolded counterpart, although such activities were reduced by quaternization (32).

In another study, Ryou et al. (33) found that the interaction of full-length recombinant mouse PrP with plasminogen can be blocked by a specific anti-kringle antibody and lysine. By blocking the binding site with lysine, the PrP^{Sc} cannot replicate; therefore, it cannot cause misfolding of the normal PrP^{C} proteins. Recently, Ryou et al. (34) found that lysine and PLK eliminate PrP^{Sc} in prion-infected cell culture models. They also showed that treatment of prion-infected mice with intravenous and intraperitoneal doses of PLK prolonged the onset of incubation time and decreased the PrP^{Sc} levels of mouse brains (34). A frequent stumbling block in using PLK is the toxicity of the compound. Recently Hunter and Moghimi (35) found that PLK can induce apoptosis in vivo mediated by the mitochondria.

Purpose of Project

The major drawback of PLK for treatment in animal models of prion disease is the toxicity associated with PLK. The purpose of this project was to examine the anti-prion activity and cytotoxicity of lysine-based compounds, PLKs conjugated with polyethylene glycol (PEG), designated PEG-PLKs. A form of PEG-PLK (PEG-

PLK 66) was tested in vitro. The cell culture model used for this study was ScN2a, a mouse cell line infected by RML scrapie prions. RML prions were adapted to mice from an original sheep scrapie pool (36). PLK 65 was used as a control in this evaluation. The objective of this study was to develop an effective lysine-based strategy for the treatment of prion disease in vitro.

Another approach for treating scrapie in vitro with PDK was also tested. For this project, PLK was compared with PDK. Ryou et al. (37) completed previous research using the compound quinacrine. Usually quinacrine was used in a solution made of two stereoisomers, (S)-quinacrine and (R)-quinacrine. When these two isomers were used separately, (S)-quinacrine had increased anti-prion activity compared to (R)-quinacrine and the mixture of both (37). Cytotoxicity of the cells remained the same whether it was (S)-quinacrine, (R)-quinacrine, or the mixture (37). Therefore, similar results should be achieved using PLK and PDK, due to the fact that sterioisomers of a bioactive compound could exhibit differential anti-prion activity.

Chapter 2 Materials

The scrapie-infected N2a (ScN2a) cell line used in this study was established in Dr. Chongsuk Ryou's laboratory and maintained for the past seven years by exposing N2a cells purchased from ATCC (American Tissue and Culture Collection) to RML (Rocky Mountain Laboratory) prions from Chandler isolates. The orginal cell line was established by Dr. Stanley Prusiner at University of California, San Francisco (UCSF) (38). Therefore, ScN2a is a murine cell line that propagates sheep scrapie prion cells originated from infected sheep in the United Kingdom.

Compounds used in this experiment were provided by academic collaborators and commercial vendors. Dr. Y. Bae, from the College of Pharmacy at the University of Kentucky provided the PEG-PLK 66. PLK 65, PLK 30-70, and PDK 30-70 were purchased from Sigma Aldrich. PLK 52 and PDK 52 were purchased from Almanda Polymers. PEG-PLK 66 and PLK 65 molecules are shown in Figure 2. One PEG molecule is added to each PLK molecule to create PEG-PLK66 in an attempt to make the compound less toxic, but still retain the anti-prion activity. This addition to the PEG-PLK 66 compound increased its molecular weight; therefore to have an accurate comparison, molar weights were used when calculating treatment amounts versus µg/mL. Figure 3 shows the molecular structure for PDK and PLK. These two compounds are stereoisomers.

The primary antibodies used in this study were monoclonal anti-PrP Hum-P antibody (obtained from UCSF) and anti-actin Ab-5 antibody (Lab Vision Corp).

Hum-P is a chimeric human-mouse Fab fragment of the anti-PrP antibody (39). The secondary antibodies used in this study were goat anti-human Fab and goat anti-mouse IgG conjugated with horseradish peroxidase (Pierce).

Standard materials were used for cell culture, bicinchonic acid assay kit (BCA, Thermo Scientific), protein digestion, western blot, and densitometry analysis. More information about these materials is recorded in Chapter 3 and Appendix III.

Chapter 3 Methods

This research project was initiated to evaluate PEG-PLK 66/ PLK 65 and PLK/ PDK for both cytotoxicity and anti-prion activity. Cytotoxicity evaluation was completed with MTT assay. Evaluation of anti-prion activity evaluation was completed with BCA analysis, PK digestion, western blot, and densitometry. ScN2a cells were used for cell culture.

The PEG-PLK 66/ PLK 65 experiment was a preliminary experiment. There was a small amount of PEG-PLK 66 to test, so only one MTT assay and one Western Blot were completed. There was not enough compound to do the assays in triplicate.

The stereoisomer experiment was completed using PLK and PDK. These compounds are the same chemically, but are structurally different. This allows the compounds to work on the same material, yet in different ways.

Cell Culture

All cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM, high glucose) containing 10% fetal bovine serum (FBS), 1% glutamax, and 1% streptomycin/penicillin (Invitrogen). Cultures were maintained at 37ºC with 5% CO_2 atmosphere and saturated humidity. Cells were maintained in a 60 mm cell culture dish (Corning) and were passed 1:10 weekly. After complete removal of old media, cells were washed in Delbecco's Phosphate Buffered Saline 1x (DPBS, Sigma-Aldrich) to remove residual media that could inactivate the trypsin (Sigma-Aldrich). DPBS was completely removed and trypsin (0.5 mL) was added to the cells to

release them from the plate. After incubation at 37°C for five minutes, media (5 mL) (Appendix III) was added to the dissociated cells. An aliquot of this mixture was placed in a new tissue culture dish and cultured under described conditions.

Cytotoxicity Evaluation

Cytotoxicity evaluations were measured with MTT assay. MTT is metabolized by the cell mitochondria and changed into formazan. The change of MTT to formazan is indicated by the yellow MTT changing to purple. If the purple color does not appear, then MTT was not metabolized, indicating that no live cells are present.

Standard cell culture techniques were used to maintain the cells, with media and treatment being changed on day three and cells collected on day six. After media was completely removed, 1 mL of MTT solution (Appendix III) was placed in each well of a 12 well plate to confirm if cells remained. Confirmation was assured when the cells turned purple. The plate was incubated at 37ºC for two hours. The media was removed from the cells and the dye was solubilized with 1 mL of acidic isopropanol. After pipetting, the solubilized dye was placed in a labeled Eppendorf tube and microcentifuged at 13,000 rpm for two minutes at 4°C. One hundred microliters of the supernatant was placed in each of the 96 well plates, in triplicate for determining the mean and standard deviation. Absorbance of converted dye was measured at a wavelength of 595 nm with the background absorbance measured at 640 nm using a Beckman Coulter DTX 880 Multi-mode Detector.

Anti-prion Activity Evaluation

Cell Lysate

Standard cell culture techniques were used to maintain the cells, with media and treatment being changed on day three and cells collected on day six. On day six, cells were washed with DPBS and cell lysis buffer (Appendix III) (500 μL) was added. After the cells were removed from the plate, the cell lysis buffer was collected into labeled eppendorf tubes. The tubes were micro-centrifuged at 7000 rpm at 4°C for two minutes. Supernatant was placed into a new Eppendorf tube and protein amounts were determined through a BCA assay.

BCA Protein Assay

Bovine Serum Albumin (BSA) (0-50 μg/mL) was used as a standard to compare the BCA protein results and confirm protein data. The standard assay uses commercially available BSA (BSA) that is certified to be at a concentration of 2 mg/ml. BCA uses the biuret reaction in which chelate copper reacts with protein in an alkaline environment. To form a light blue complex of the reduced cation, bicinchoninic acid reacts with the cation and an intense purple-color forms, indicating the chelation of two BCA molecules and one cuprous ion. The color darkens as the amount of protein increases in the sample.

Protease K digestion

Samples were digested with PK, because PrP^{Sc} is resistant to PK. PrP^{C} and other peptides in the sample are not resistant to PK digestion; therefore only PrP^{Sc} should remain after digestion.

One milligram from each sample of cell lysate was digested with PK (20 µg/µL). Samples were incubated for one hour at 37ºC with shaking (300 rpm). Phenylmethanesulfonyl fluoride (PMSF) (2mM) was added and samples were micro centrifuged at 14,400 x on a CTT525221 Fischer Scientific micro-centrifuge at 4ºC for two hours. The pellet was resuspended with 1X sample loading buffer (SLB, Appendix III). The undigested samples used 40 µg of cell lysate mixed with 4X SLB (5 µL). Both samples were boiled at 100ºC for 10 minutes.

Equal protein amounts of cell lysate were used for western blotting. For undigested sample analyses, 40 µg of protein was used; 1.0 mg of protein was used for digested samples. More protein was needed for digested samples because the absolute amount of PrP^{Sc} in 40 µg of cell lysate was below detection limits.

Western Blotting and Densitometry

Western Blotting Gel

A 12% poly-acrylamide resolving gel was used as a separation matrix for PrP^{Sc} for the western blotting analysis. A 5% stacking gel was added to concentrate the sample into an ultrathin zone, where the polypeptides can enter the resolving gel based on their size and shape.

Western Blotting Analysis

After PK digestion, the samples were analyzed through western blotting. A protein marker (5 µL) (Precision Plus Protein Dual Color Standards, Bio-Rad) was loaded into the first well of each 12% poly-acrylamide gel. Twenty-five microliters of each sample were loaded in wells 2-15. Running buffer (Bio-Rad Laboratories, Inc.) was poured over the samples; electrophoresis was carried out at 80 volts for 15 minutes and 100 volts for two hours. After the running cycle, the samples were transferred from the gel to an immobilon transfer membrane (Fisher Scientific) at 90 volts for one hour, using a transfer buffer (Bio-Rad Laboratories, Inc.). The membrane was then blocked with a 5% milk bath for 1 hour before antibodies were introduced. The primary anti-body, Hum-P, was diluted 1:5000 with Tris Buffer Saline with Tween-20 (TBST, Appendix III) and shaken with the covered blocked membrane overnight at 40ºC. The next day, the membrane was washed four times with TBST. The secondary anti-body, goat anti mouse, was shaken at 25ºC for one hour. The membrane was washed again four times with TBST.

After the fourth washing, the membrane was developed on Fujifilm FLA500 using an ECL kit (GE Healthcare). The ECL kit was a non-radioactive method that detected antigens immobilized onto membranes. It used horseradish peroxidase (HRP) combined with a secondary anti-body. These combined with luminal, the chemiluminescent substrate, generated the signal that was captured on film. Doc-IT software (UVP) was used to analyze the resulting data for densitometry analysis.

Chapter 4 Results

Tables are located in Appendix I and all figures are located in Appendix II of this thesis.

Cytotoxicity of PEG-PLK 66 and PLK 65: Preliminary Study

Cell viability was measured by MTT assay. MTT assay results showed PEG-PLK 66 was less toxic to cells compared with PLK 65 (Table 1, Figure 5). For PEG-PLK 66, 50% of cells survived at a concentration of 15 μM, whereas PLK 65 killed all cells at a concentration of 10 μM.

Anti-Prion Activity of PEG-PLK 66 and PLK 65: Preliminary Study

PK digestion followed by western blotting and densitometry were used to analyze the anti-prion activity of PEG-PLK 66 and PLK 65. The protein content of the cell lysate treated with PEG-PLK 66 and PLK 65 was quantified by BCA assay (Table 2). Although PLK 65 was shown to have better anti-prion activity than PEG-PLK 66, PEG-PLK 66 still reduced PrP^{Sc} to undetectable levels (Table 3, Figure 6). PEG-PLK 66 required 10 times higher concentrations than PLK 65 to reduce the same levels of PrP^{Sc} to zero or near zero, but these concentrations were still within the non-toxic concentrations determined by MTT assay. Actin blotting confirmed that equal amounts of protein in the cell lysates were used for western blotting.

Cytotoxicity Evaluation of PDK and PLK

Cell viability was measured by MTT assay. PDK was more toxic than PLK. While PLK 30-70 had a LC_{50}=10μg/mL (concentration at which 50% of the cells

were killed), there was complete cell death at 20 μg/mL. PDK 30-70 had a LC_{50}=3μg/mL, while 10 μg/ml concentration complete cell death occurred (Table 4, Figure 7). Although PDK 30-70 and PLK 30-70 are composed of D- and L- lysine polymers with molecular weights between 30 and 70 kDa, the molecular weight of the PLK 30-70 used for this experiment was greater than that of PDK 30-70 according to manufacturer's instructions. Therefore, PLK 52 and PDK 52 were obtained because they have the same polymerization degrees and molecular weights. By using isomers of identical molecular weights, the efficacy of PDK and PLK can be accurately compared. The results showed that PLK 52 had a LC_{50}=31 μg/mL and did not have complete cell death at 50 μg/mL (Table 5, Figure 8). PDK 52 had a LC_{50}=3.5μg/mL and complete cell death occurred at 10 μg/mL concentration.

Anti-Prion Activity of PDK and PLK

Western blotting of PK-digested cell lysate was used to determine anti-prion activity of PDK and PLK. After protein quantification using BCA assay (Table 6, 8), equal protein amounts were used in western blotting. PDK was shown to have better anti-prion activity than PLK (Table 7, 9, Figure 9, 10). PDK 30-70 and PDK 52 removed PrP^{Sc} to undetectable levels at a concentration of 0.25 μg/mL. PDK 30-70 and PLK 52 had an IC_{50}=0.13μg/mL (concentration at which prions were reduced to 50%). PLK 30-70 had an IC_{50}=1.25μg/mL, while PLK 52 had an IC_{50}=0.95 μg/mL. Neither PLK 30-70 nor PLK 52 fully removed all the prions from the samples at 2μg/mL.

Chapter 5 Discussion

The effect of PEGylation on PLK

PEGylation reduced the anti-prion activity of PLK, although prion levels were still reduced to undetectable levels. The concentrations PEG-PLK required to reduce prions to undetectable levels were below the cytotoxic level determined by MTT assay. The reason PEGylation interfered with anti-prion activity could be because PEG could hinder PLK compounds preventing them from reaching their target. In general, PEG is often used with toxic compounds to decrease toxicity and increase the availability of the compound for treatment. (40) PEG is soluble in water and protects PLK when attached to it. With this protection, PLK was able to reach the site for PrP conversion to PrP^{Sc} and inhibit it. By attaching PEG to PLK, PLK could stay in the cellular system for longer periods of time. These results demonstrate that toxicity of PLK is improved, but the anti-prion activity is decreased by PEGylation. A previous study also documented that PEG reduces toxicity while interfering with the availability of compounds (41).

In this experiment, treated cells were incubated with increasing molar concentrations of PLK so that the molecular weight of the PEG would not skew the results. This allowed for equal concentrations of the PLK moiety to be applied to the cells.

The effect of PLK stereoisomers

Stereoisomers of PLK had improved anti-prion activity as well as increased cytotoxicity. Concentrations at which anti-prion activity was improved were still well below the toxic levels determined by the MTT assay. Increased cytotoxicity of PDK (LC_{50}=3μg/mL vs PLK's LC_{50}=31μg/mL) to the cells could be due to the difference in configuration of PLK and PDK. Because the biological systems of mammalian species usually metabolize amino acids in L-forms, the enzymes in the cell system do not recognize D-lysine as they would L-lysine. This allows the PDK to stay in the system longer with a possible build-up of its concentration, when given at the same concentrations of PLK. This difference increases the cytotoxicity of PDK, while also increasing the anti-prion activity. Due to the slight difference of the PDK structure, this allowed it to by-pass the mechanisms that the cells had in place for breaking down PLK, therefore allowing more efficient use of the PDK for anti-prion activity. This was shown through the PDK IC_{50}=0.13μg/mL vs. the PLK IC_{50}=1.25μg/mL. Therefore, PDK becomes more effective and inhibits prions below cytotoxic levels.

Future Applications

There is potential for use of these compounds in treatment of prion disease, but much research must be done to ensure that any introduction of new feed additives, injectable drugs, or vaccines are safe for use in live animals, particularly those designated for human consumption. There are many steps between in vitro studies

such as these, and clinical application, including rigorous and highly regulated studies using animal models. Additional studies are needed to examine cytotoxicity as well as anti-prion activity in vivo. Research is needed on how the compounds distribute through the animal and cellular system, as well as determining the best method for getting the compounds into the animal. Once these studies are completed through good laboratory practices (GLP), animal studies, and clinical 1, 2, and 3 stages, lysine-based polymer compounds (whether used in modified form or as a stereoisomer) may be a good lead for a pre-clinical compound to be used as a therapy against prion disease.

Some potential uses for this research would be to develop a PEGylated stereoisomer (PEG-PDK) to decrease PDK's toxicity while improving anti-prion activity. This modified version of PLK would, in theory, stay in the body longer and need lower treatment concentrations and dosages than standard PLK. There is also the possibility that the compound could be used for prophylactic purposes.

Chapter 6 Conclusion

Modification of PLK with PEG reduced cytotoxicity and anti-prion activity. Although PEG-PLK reduced anti-prion activity, prions were still removed to an undetectable level at concentrations below the toxic concentration level determined by the cytotoxicity experiment. PDK had an increased effect on prions as well as increased cytotoxicity when compared to PLK. Although PDK was more toxic, it also removed prions at a non-toxic concentration. This study provides useful data for future strategies to develop lysine-based compounds that combat prion diseases.

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Appendixes

Appendix I Tables

Table 1. Comparison of cytotoxicity assay results for PEG-PLK 66 and PLK 65, measured with MTT assay. These results show the percentage of surviving cells at each μM concentration of PLK and PEG-PLK.

Compound	Concentration (μM)	Absorbance at 595 nm[A]	Normalized Data (% of cells that survived)[B]
PLK 65	**1**	0.60	100
	3	0.22	36.21
	10	0.00	0.45
	30	0.00	0.62
PEG-PLK 66	**1**	0.88	100
	3	0.63	71.68
	10	0.50	56.85
	30	0.17	19.40

[A] Absorbance measurement of color of the sample at 595 nm.

[B] Normalized Data is the percent of cells that survived the treatment.

Table 2. Protein analysis for PEG-PLK 66 and PLK 65, measured with BCA. BCA gives total average protein amount for each sample. 40 µg of protein was used for western blotting of undigested samples. 1 mg of protein was used for western blotting of digested samples.

PLK 65			PEG-PLK 66		
Concentration (µM)	**40 µg protein[A]**	**1 mg protein[B]**	**Concentration (µM)**	**40 µg protein[A]**	**1 mg protein[B]**
0	18.4	460.1	**0**	20.8	518.9
0.05	18.2	455.3	**0.1**	21.6	540.1
0.1	10.7	267.4	**1**	17.3	433.3
0.25	13.3	332.6	**10**	19.8	494.6
0.5	13.3	331.6			
1	13.1	326.6			

[A] µg amount per undigested sample.

[B] µg amount per digested samples.

Table 3. Densitometry results for PEG-PLK 66 and PLK 65, measured with western blot analysis. This table gives the average normalized mean of densitometry and the standard deviation at each µM concentration. Densitometry measures the band intensity for each of the samples.

PLK 65			PEG-PLK 66		
Concentration (µM)	Mean[A]	Standard Deviation	**Concentration (µM)**	Mean[A]	Standard Deviation
0	100	0	**0**	100	0
0.05	23.33	1.53	**0.1**	5	3
0.1	8.67	0.58	**1**	15.33	1.15
0.25	11	0	**10**	0	0
0.5	0.1	0			
1	0	0			

[A]Average normalized values for the anti-prion activity of the compound.

Table 4. Comparison of cytotoxicity assay results for PLK 30-70 and PDK 30-70, measured with MTT assay. These results show the percentage of surviving cells at each µM concentration of PLK and PEG-PLK.

Compound	Concentration (µg/mL)	Absorbance at 595 nm[A]	Normalized Data (% of cells that survived)[B]	Standard Deviation
PLK 30-70	**0**	1.92	100	0.12
	1	1.56	81.15	0.11
	2	2.01	104.67	0.18
	4	1.51	78.29	0.12
	10	0.98	51.29	0.07
	20	0.02	1.00	0.00
	50	0.00	0.21	0.00
PDK 30-70	**0**	1.92	100	0.12
	1	1.99	103.43	0.13
	2	1.98	103.20	0.11
	4	0.07	3.63	0.00
	10	0.00	0.18	0.00
	20	0.00	0.28	0.00
	50	0.01	0.35	0.00

[A] Absorbance measurement of color of the sample at 595 nm.

[B] Normalized Data is the percent of cells that survived the treatment.

Table 5. Comparison of cytotoxicity assay results for PLK 52 and PDK 52, measured with MTT assay. These results show the percentage of surviving cells at each µM concentration of PLK and PEG-PLK.

Compound	Concentration (µg/mL)	Absorbance at 595 nm[A]	Normalized Data (% of cells that survived)[B]	Standard Deviation
PLK 52	**0**	1.48	100	0.05
	1	1.13	76.37	0.04
	2	1.38	93.07	0.05
	4	1.37	92.59	0.11
	10	1.21	82.09	0.10
	20	1.06	71.74	0.04
	50	0.19	12.76	0.01
PDK 52	**0**	1.01	100	0.06
	1	1.19	117.79	0.02
	2	0.97	96.38	0.05
	4	0.30	29.47	0.02
	10	0.00	0.16	0.00
	20	0.00	0.33	0.00
	50	0.00	0.43	0.00

[A] Absorbance measurement of color of the sample at 595 nm.

[B] Normalized Data is the percent of cells that survived the treatment.

Table 6. Protein analysis in PLK and PDK 30-70, measured with BCA. BCA gives total average protein amount for each sample. 40 µg of protein was used for western blotting of undigested samples. 1 mg of protein was used for western blotting of digested samples.

PLK 30-70			PDK 30-70		
Concentration (µg/mL)	**40 µg of protein[A]**	**1 mg of protein[B]**	**Concentration (µg/mL)**	**40 µg of protein[A]**	**1 mg of protein[B]**
0	12.7	318.3	**0**	13.7	342.7
0.25	19.0	475.3	**0.25**	17.9	447.9
0.5	13.4	334.9	**0.5**	11.8	294.7
0.75	14.2	356.0	**0.75**	11.7	291.2
1	15.5	387.10	**1**	12.6	314.6
1.5	12.9	321.8	**1.5**	12.7	317.3
2	14.2	354.1	**2**	13.4	335.5

[A] µg amount per undigested sample.

[B] µg amount per digested samples.

Table 7. Densitometry results for PLK and PDK 30-70, measured with western blot analysis. This table gives the average normalized mean of densitometry and the standard deviation at each µM concentration. Densitometry measures the band intensity for each of the samples.

PLK 30-70			PDK 30-70		
Concentration (µg/mL)	**Mean[A]**	**Standard Deviation**	**Concentration (µg/mL)**	**Mean[A]**	**Standard Deviation**
0	100	0	**0**	100	0
0.25	114.15	90.57	**0.25**	0	0
0.5	161.93	153.64	**0.5**	0	0
0.75	102.56	68.80	**0.75**	0	0
1	66.70	41.05	**1**	2.17	3.75
1.5	35.09	12.78	**1.5**	3.60	6.24
2	23.78	12.83	**2**	2.00	3.41

[A] Average normalized values for the anti-prion activity of the compound.

Table 8. Protein analysis in PLK and PDK 52, measured with BCA. BCA gives total average protein amount for each sample. 40 µg of protein was used for western blotting of undigested samples. 1 mg of protein was used for western blotting of digested samples.

PLK 52			PDK 52		
Concentration (µg/mL)	**40 µg of protein[A]**	**1 mg of protein[B]**	**Concentration (µg/mL)**	**40 µg of protein[A]**	**1 mg of protein[B]**
0	10.4	260.8	**0**	12.0	301.0
0.25	9.7	243.0	**0.25**	10.4	258.6
0.5	9.9	247.0	**0.5**	12.2	303.9
0.75	9.8	244.8	**0.75**	11.6	291.0
1	8.0	224.1	**1**	10.5	262.6
1.5	13.3	332.2	**1.5**	9.7	241.8
2	10.3	257.3	**2**	10.9	272.4

[A] µg amount per undigested sample.

[B] µg amount per digested samples.

Table 9. Densitometry results for PLK and PDK 52, measured with western blot analysis. This table gives the average normalized mean of densitometry measured and the standard deviation at each µM concentration. Densitometry measures the band intensity for each of the samples.

PLK 52			PDK 52		
Concentration (µg/mL)	**Mean[A]**	**Standard Deviation**	**Concentration (µg/mL)**	**Mean[A]**	**Standard Deviation**
0	100	0	**0**	100	0
0.25	79.91	53.71	**0.25**	0	0
0.5	50.03	49.67	**0.5**	0	0
0.75	61.17	25.81	**0.75**	0	0
1	45.75	36.14	**1**	0	0
1.5	28.56	40.38	**1.5**	3.00	4.24
2	32.13	14.72	**2**	0	0

[A] Average normalized values for the anti-prion activity of the compound.

Appendix II Figures

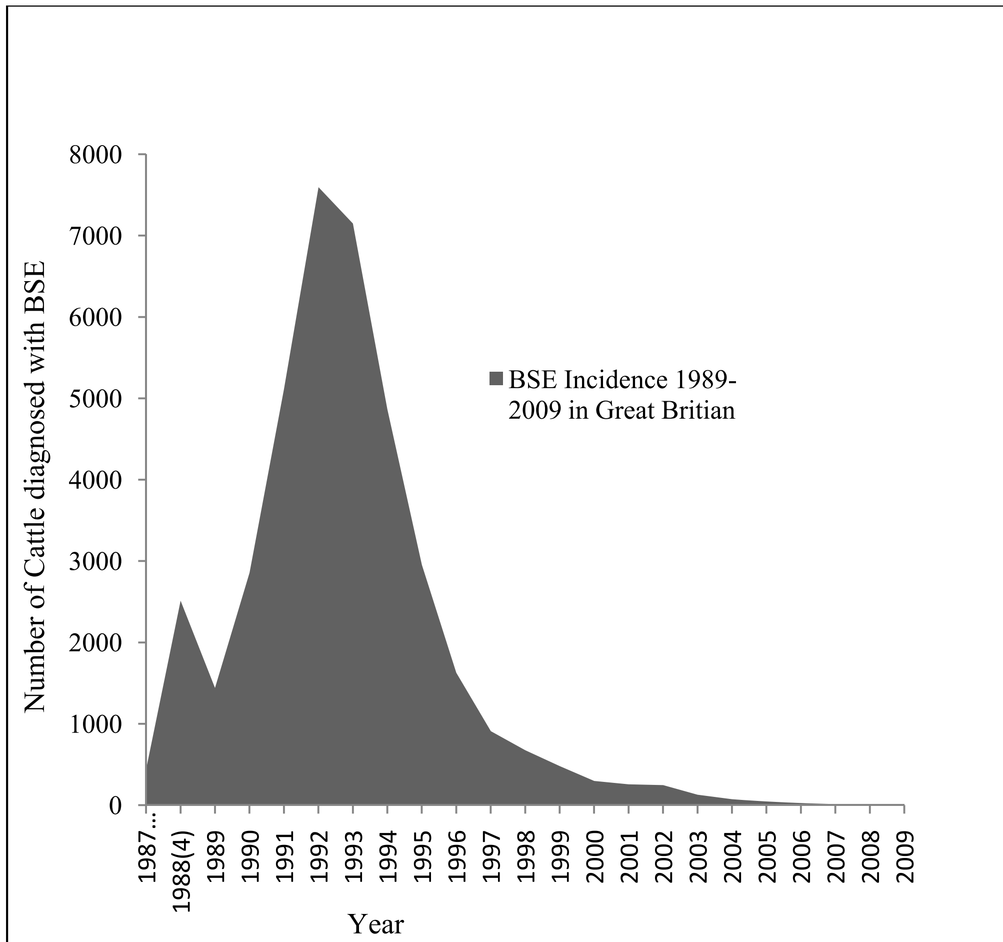


Figure 1. BSE statistics in the United Kingdom, by month from the onset of the disease. Data taken from World Animal Health Organization (OIE).

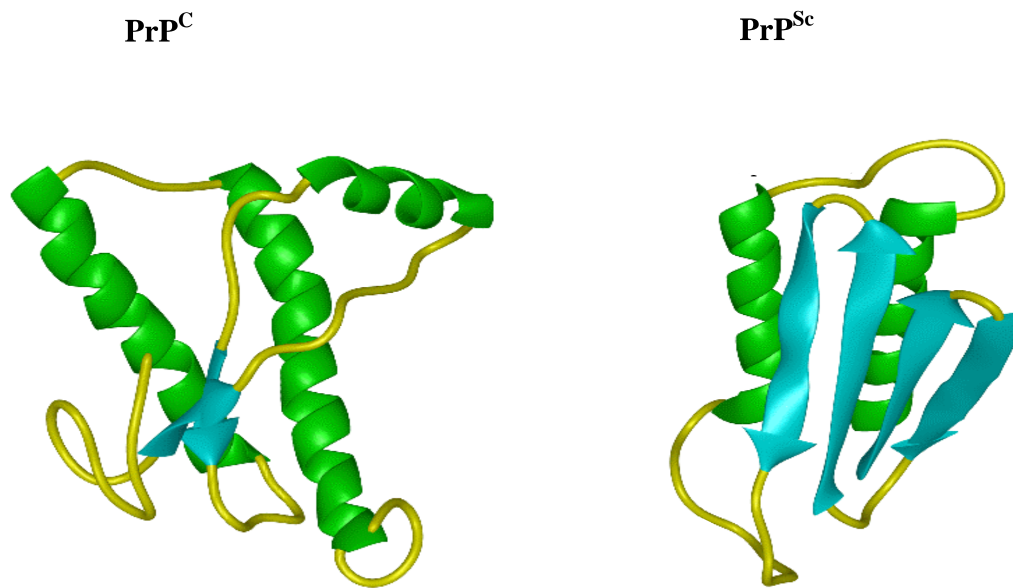
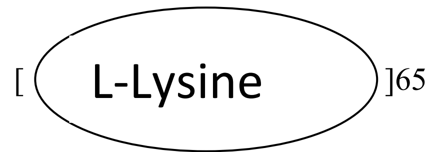


Figure 2. Molecular structure comparing normal PrP^{C} and scrapie PrP^{Sc}. The following pictures exhibit a normal PrP^{C} and a PrP^{Sc} (Scrapie infected PrP^{C}) protein. Normal PrP^{C} has more α-helices than β-sheets. PrP^{Sc} has more β-sheets than α-helices. This picture was used with modification from www. cmpharmucsf.edu.

PLK65



PEG-PLK66

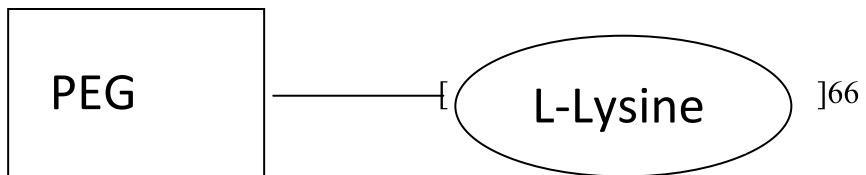


Figure 3. Drawing comparing PLK 65 and PEG-PLK 66.

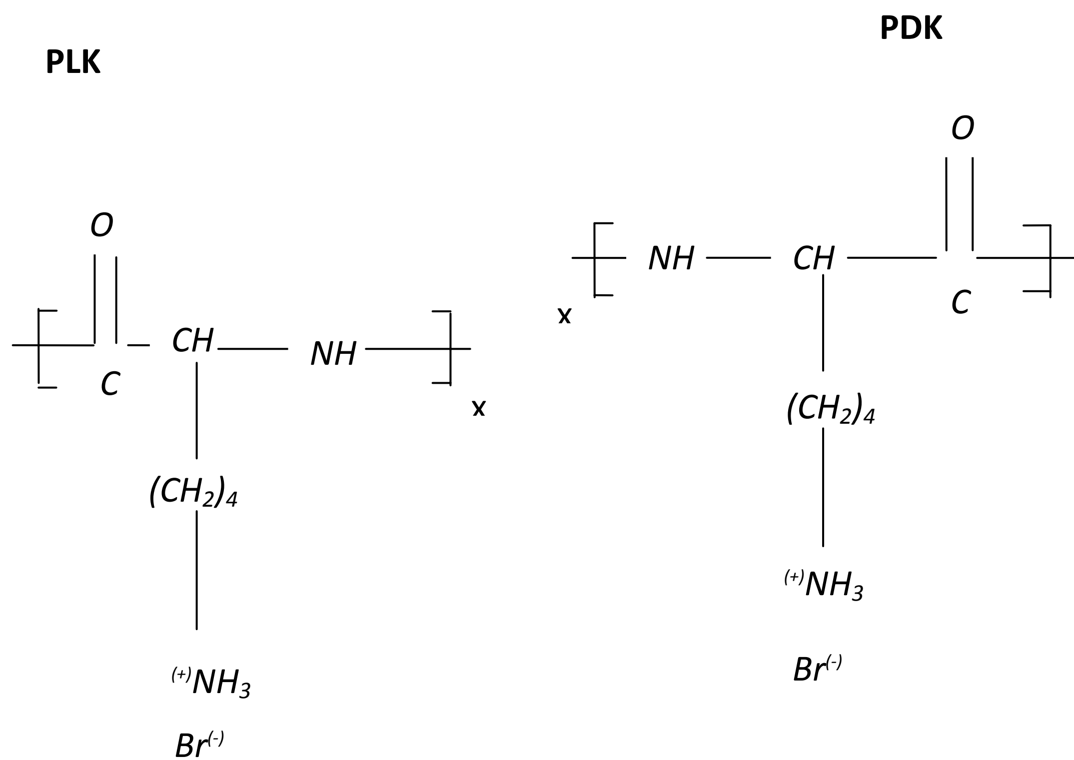


Figure 4. Molecular structure of PDK and PLK.

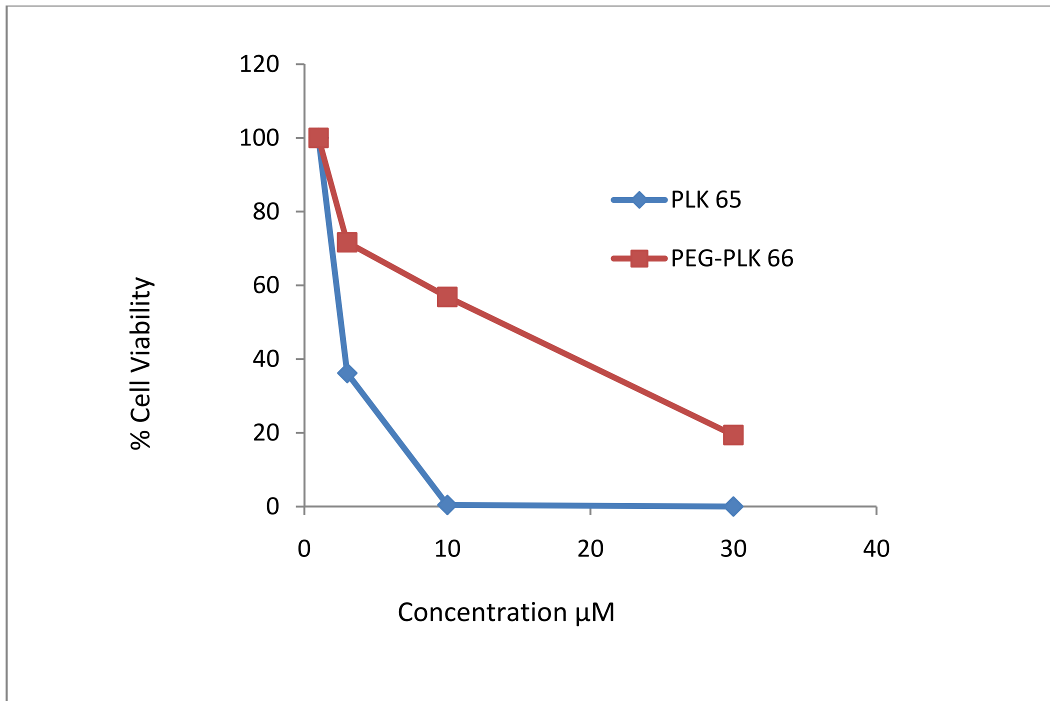


Figure 5. Cytotoxicity results comparing PEG-PLK 66 and PLK 65 in µM, measured with MTT assay. Cytotoxicity results were measured with MTT assay. PEG-PLK 66 is less toxic than PLK 65 in vitro.

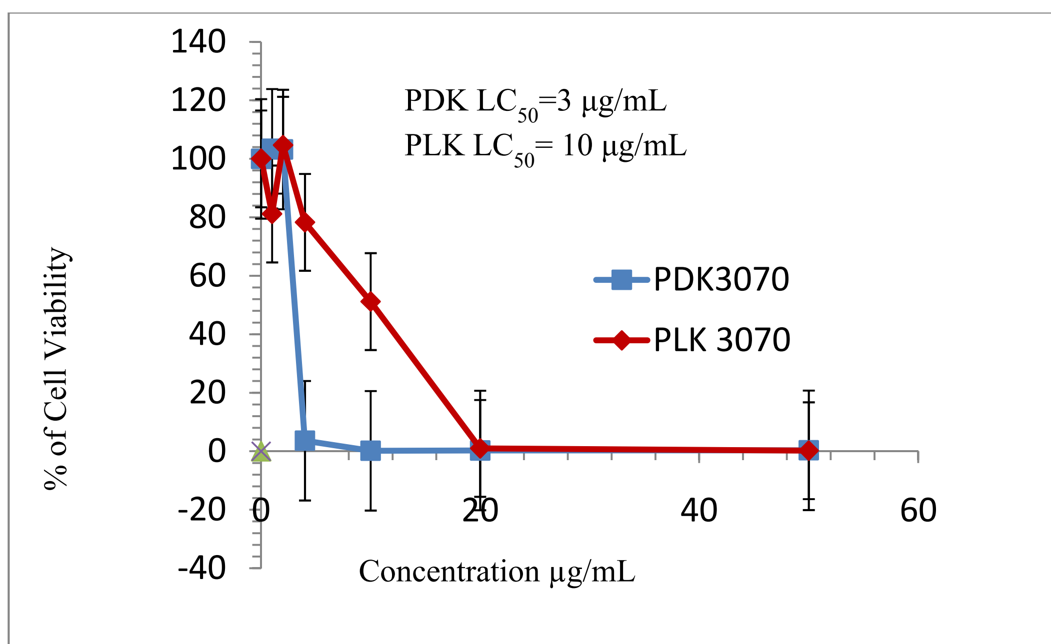


Figure 7. Cytotoxicity results comparing PLK and PDK 30-70, measured with MTT assay. Error bars for cytotoxicity results indicate standard deviation. LC_{50} is the concentration at which 50% of the cells died.

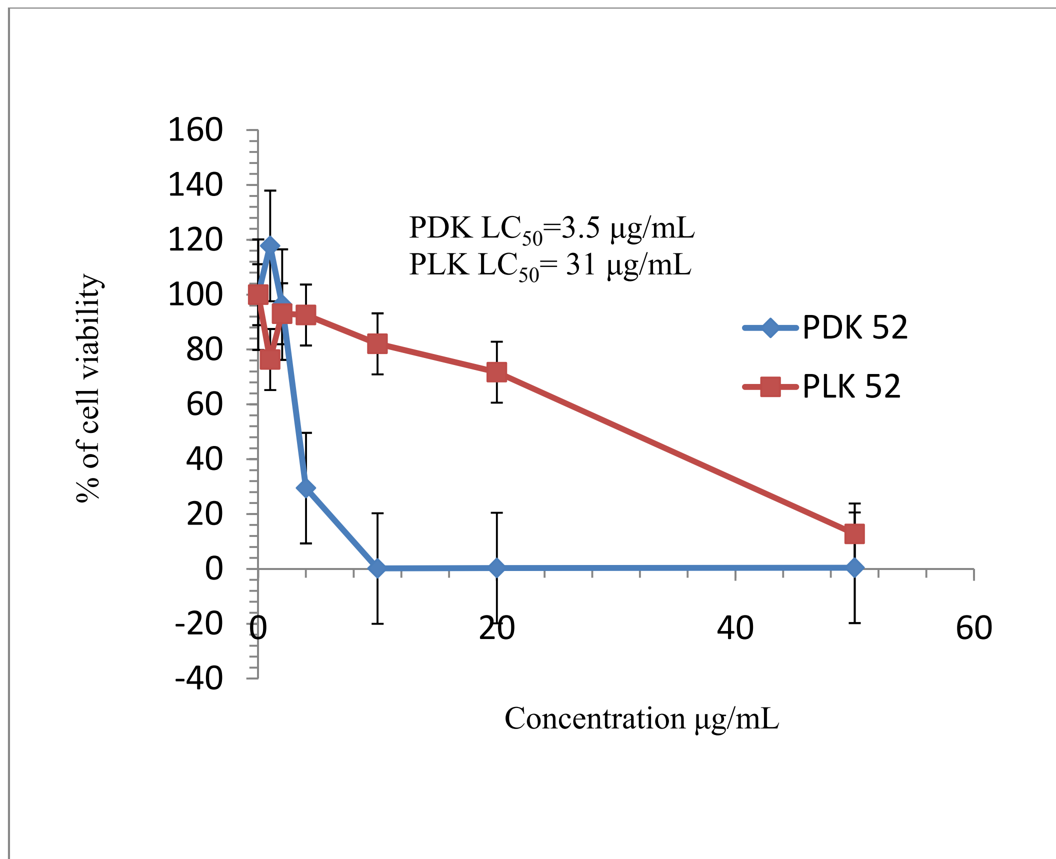


Figure 8. Cytotoxicity results comparing PLK and PDK 52, measured with MTT assay. PLK is less toxic than PDK. Error bars for cytotoxicity results indicate standard deviation. LC_{50} is the concentration at which 50% of the cells died.

Appendix III Formulations

Growth media

500 mL Delbecco's modified Eagle medium)1X (DMEM) (Sigma-Aldrich)

50 mL fetal bovine serum (FBS) (Sigma-Aldrich)

5 mL penicillin/ streptomycin (pen/strep) (Sigma-Aldrich)

5 mL Glutamax (Sigma-Aldrich)

Cell lysate buffer

2% 1 M Tris hydrochloride (HCL) (Sigma-Aldrich)

3.75% 4 M Sodium Chloride (NaCl) (Sigma-Aldrich)

5% 10% Nonidet P 40 (NP-40) (Thermo Scientific)

5% 10% Sodium deoxycholate (Doc) (Sigma-Aldrich)

84.25% distilled water

5% Stacking Gel

2.85 mL	distilled water
850 uL	100% Acryl/ Bisacryl
1.25 mL	0.5M Tris HCL pH 6.8 (stacking gel buffer)
50 uL	10% SDS
25 uL	10% APS
5uL	100% TEMED

Combine all chemicals and mix until all solutions are dissolved. Replace water layer of 12% agrose gel with 5% stacking gel mixture. Insert comb.

12% Acryl/Bisacryl Gel

5.1 mL	distilled water
6.0 mL	Acryl/ Bisacryl (Bio-Rad)
3.75 mL	1.4M Tris HCL pH 8.8 (resolving gel buffer)
150 uL	10% sodium dodecyl sulfate (SDS) (Sigma-Aldrich)
75 uL	10% ammonium persulfate (APS) (Sigma-Aldrich)
1.5 uL	100% Tetramethyl-ethylenediamine ReagentPlus 99% (TEMED) (Sigma-Aldrich)

Combine all chemicals and mix until all solutions are dissolved. Fill gel mold 80% with mixture. Top with distilled water to keep moist while gelling.

Running buffer

100 mL	10X Tris/ Glycine/ SDS (TGS) (Bio-Rad)
900 mL	distilled water

Transfer buffer

100 mL	10X Tris/ Glycine (TG) (Bio-Rad)
200 mL	Methanol (MeOH) (VWR)
700 mL	distilled water

Blocking buffer

1 g	dry milk
20 mL	TBST

Tris Buffer Saline with Tween-20 (TBST) wash buffer

800 mL distilled water

8.0 g NaCl

0.2g potassium chloride (KCl) (Sigma-Aldrich)

3 g Tris base (Sigma-Aldrich)

500 uL 100% Tween-20 (Sigma-Aldrich)

Combine NaCl, 400 mL distilled water, 0.2 g KCl, 3g Tris Base, and 500 uL of Tween-20. Stir on magnetic plate until solids dissolve and pH to 7.0. Add remaining water unil 1 L volume and stir.

MTT Solution

1 mL 5ug/mL Thiazolyl Blue Tetrazolium Bromide (MTT) (Sigma-Aldrich)

9 mL DMEM

Acidic Isopropanol

uL 0.04M HCL (Bio-Chemika)

Vita

Karen Jackson was born in Kingsport, Tennessee to Lester and Cathy Strong. She had two sisters, Emily and Katrina. Karen grew up on a small family farm in Church Hill, TN where she raised goats, horses, cattle, and chickens. She attended Elementary and Secondary school in Church Hill, TN. In 2001, she graduated from Berea College, Berea, KY with a B.S. in Agriculture and Natural Resources. While at Berea College, she started the goat program, completed a hair sheep internship at University of the Virgin Islands in St. Croix, Virgin Islands, and a goat internship at Langston University, Langston, OK. After graduating college, she married Shaun Jackson in 2002, and held a split extension position in Lawrence County, TN until 2007. In 2005, she started on her Master's program in Agriculture and Natural Resources Systems Management with a concentration in Agricultural Education and Leadership at The University of Tennessee at Martin. In 2007, Karen and her family moved to Lexington, KY where she joined Laboratory Animal Resources and received her Laboratory Technologist Certification while completing her Master's Degree. Currently, she resides in Lexington, KY with her husband and 4 year old son.