DISTINCT TAU STRAINS:
EXPLORING VARIABILITY IN CELL UPTAKE AND SEEDING

by

WILLIAM LLOYD PRUEITT

DISSERTATION

Presented to the Faculty of the Medical School of
The University of Texas Southwestern Medical Center
In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF MEDICINE WITH DISTINCTION IN RESEARCH

The University of Texas Southwestern Medical Center
Dallas, TX

ACKNOWLEDGMENTS

I would like to thank Drs. Barbara Stopschinski, M.D. and Marc Diamond, M.D. for their invaluable help and guidance throughout this project. I would also like to thank Dr. Lukasz Joachimiak, Ph.D. for his feedback while serving on my thesis committee. Finally, I would like to thank The University of Texas Southwestern Medical Student Summer Research Fellowship for their financial support of this work.

ABSTRACT
DISTINCT TAU STRAINS:
EXPLORING VARIABILITY IN CELL UPTAKE AND SEEDING

WILLIAM LLOYD PRUEITT
The University of Texas Southwestern Medical Center, 2019
Supervising Professor: Marc Diamond, M.D.

Background: Tauopathies are neurodegenerative diseases characterized by the pathological aggregation of the microtubule-associated protein tau in neurons and glia. These conditions are incurable, progressive, and deadly. Alzheimer's Disease, the most common tauopathy, affects more than 30 million people worldwide and will afflict more than 120 million by 2050. Evidence suggests that tau aggregates spread pathology as do prions, infectious proteins that transmit a pathologic conformation to native proteins via disease-specific conformers (strains). Various tau strains have been identified which propagate stably in cultured cells over many generations. Additionally, evidence shows that tau aggregates enter cells through heparan sulfate proteoglycan (HSPG) mediated macropinocytosis. However, it is unknown if: 1) different tau strains bind HSPGs uniquely or generically to trigger uptake; 2) which HSPG size and sulfation patters are important for cellular uptake of tau.

Objective: Test for differential inhibition of cellular uptake using heparin, heparinoids, and HSPG modifications; and test effects of HSPG size and sulfation patterns on binding to tau.

Methods: A "biosensor" cell line responsive to tau aggregates was used to measure intracellular tau aggregation based on fluorescence resonance energy transfer (FRET). The biosensors were HEK-293T cells which overexpress the tau repeat domain (RD) with the disease-associated P301S mutation and were tagged with cyan or yellow fluorescent proteins (RD-CFP/YFP). Cell lysate from various strains of tau was used as source material for pathologic tau seeds to induce aggregation of native tau protein within the biosensors. Lysate was incubated with heparin or heparinoids (heparin-derived molecules of varying length and sulfation patterns) for 24-hours and then added to biosensor cells in culture. When incubated in this way, heparin and heparinoids block cellular uptake of tau by preventing its binding to HSPGs. In a separate assay, lysate was added to cultured biosensor cells with CRISPR/Cas9

knockouts of important genes in the HSPG synthesis pathway. In both assays, cells were harvested 48 hours after lysate/lysate-heparinoid addition and seeding was quantified using FRET flow cytometry.

Results: All tau strains tested (DS 5, 6, 8, 9, 10, 13, 14, 15, 16, 17) were highly sensitive to heparin inhibition of seeding and most maintained a highly similar dose response (IC50 of ~100 nM). Some strains, however, showed subtle differences. At maximal heparin concentrations (200 ug/mL), noticeably higher seeding vs baseline was observed in DS 5 and 6 (17%, 9%) as compared to the other strains (<5%). When using heparinoids of 4, 8, 12, and 16 disaccharide units to inhibit tau uptake, similar patterns were seen in DS 9 and 10 (seeding reduction: dp4 = 21% vs 19%; dp8 = 27% vs 33%; dp12 = 70% vs 64%; dp16 = 63% vs 46%). Heparinoids that were desulfated at the 2-O, 6-O, and N positions also showed similar patterns of tau uptake inhibition in DS 9 and 10 (De-2-O = 65% vs 53%; De-6-O = 52% vs 25%; De-N = 35% vs 13%). Finally, seeding in HSPG genetic knockout cells was reduced substantially across strains tested in two knockout cell lines (for genes *EXT1* and *NDST1)*. Interestingly, DS 5, DS 6, and DS 15 showed less reduction than the other strains in the knockout cell lines (-38%, -50%, and -51% respectively vs roughly -65% for other strains). Finally, seeding in the third knockout cell line (*HS6ST2*) increased across all strains tested ranging from +13% to +58%.

Conclusions: Cellular uptake of many tau strains is similarly inhibited by heparin, hinting that the same heparinoid (or small molecule analog) could be used to treat diverse tauopathies. However, the unique behavior of some strains suggests that a one-size-fits-all treatment approach may not always be sufficient. Additionally, certain heparin size and sulfation patterns have specific importance for tau binding. Larger heparinoids better inhibited tau seeding (dp16 & dp12 > dp8 & dp4). Regarding sulfation patterns, the relative importance for tau binding of the sulfate moieties tested is: N-sulfation > 6-O-sulfation > 2-O-sulfation. This pattern remains consistent in recombinant tau, DS 9, DS 10, and in the genetic knockout data gathered in this project (using strains) and by others in the laboratory (using recombinant tau). Overall, this data shows many similarities and some differences in cellular uptake between strains of tau. Additional research to further characterize these differences could have important implications for understanding the diversity of tauopathies and finding unique approaches to diagnosis and treatment.

TABLE OF CONTENTS

CHAPTER 1: Introduction

Tauopathies are neurodegenerative diseases characterized by the pathological aggregation of the microtubule-associated protein tau in neurons and glia (6-9). These conditions are incurable, progressive, and deadly. Alzheimer's Disease, the most common tauopathy, affects more than 30 million people worldwide and will afflict more than 120 million by 2050 (14). A better understanding of tauopathies and the development of new therapeutic approaches is desperately needed.

Tau protein has been implicated as the proximal cause of tauopathies because mutations in the tau gene cause an autosomal dominant tauopathy (7-9) and tau filament deposition correlates closely with cognitive dysfunction and cell death in Alzheimer's Disease (6). Strong evidence suggests that tau aggregates spread pathology similarly to prions, which are infectious proteins that propagate by transmitting their misfolded state to native proteins (5). Though pathologic tau is not known to be infectious, tauopathies and prionopathies are similar in multiple other ways. First, tau aggregates can transmit a misfolded state from outside to inside a naïve cell and once within act as proteopathic seeds to induce pathologic aggregation of native tau (1,3). Second, tau pathology begins in discrete, disease-specific regions, and over time spreads to distant areas of the brain along known neural networks (10-12). Third, tau aggregates can form multiple structural conformers ("strains"), and (according to the prion model for tau) different strains may be associated with distinct tauopathies (4). A strain is a unique structural conformation of tau which can be propagated stably through many generations in cell culture and animal models (4).

Holmes et al. previously showed that recombinant tau uptake into cells is mediated by binding to heparan sulfate proteoglycans (HSPGs). HSPGs are cell surface receptors composed of a proteoglycan core that is decorated with heparan sulfate side chains which vary in the composition of their sugar chains and sulfation patterns. Tau binding to HSPG cell surface receptors triggers uptake into the cell via macropinocytosis (2).

Scientific evidence from the last decades suggests that heparin mimetics may inhibit prion pathogenesis in animal models (13). Heparin and heparinoids are sulfated sugar chains that resemble the heparin sulfate side chains of HSPGs (Figure 13) and can be used as competitive inhibitors of tau binding to HSPGs. In this way, heparin and heparinoids may prevent tau uptake into cells and halt the spread of pathology. It has not yet been determined, however, whether inhibition of uptake and seeding

by heparin and heparinoids is similar or variable across different tau strains. Additionally, it is unknown whether distinct size and sulfation patterns of the heparan sulfate side chains on HSPGs are required for tau uptake. This information has important implications for our understanding of tau pathobiology and the design of targeted therapies. If strains have varying sensitivity to heparin/heparinoids or variable binding to HSPGs with distinct size/sulfation patterns, a one-size-fits-all treatment approach across strains may be ineffective.

To answer these questions, I tested cellular uptake and intracellular seeding induced by various tau strains using heparin and different heparinoids as competitive uptake inhibitors. The importance of various HSPG size and sulfation patterns for tau binding was evaluated using distinct heparinoids. It was also examined using cells with CRISPR/Cas9 induced alterations in important genes in the HSPG synthesis pathway (*EXT1*, *NDST1*, *HS6ST2)*. *EXT1* is important for elongation of the HSPG side chain. *NDST1* and *HS6ST2* are important for modification of sulfate moieties on the HSPG side chain (N-sulfation and 6-O-sulfation, respectively).

Knowledge derived from this work is applicable across many tauopathies, furthers our understanding of tau's pathological biochemical interactions, sheds light on important size and sulfation patterns on heparin, and provides information that may aid in the development of disease-specific therapy.

CHAPTER 2: Experimental Procedures

Biosensor Cells

A "biosensor" cell line created in the Diamond lab was used in the seeding assay. The biosensors are HEK-293T cells which overexpress the tau repeat domain (RD) which contains the disease-associated P301S mutation and were tagged with cyan or yellow fluorescent proteins (RD-CFP/YFP) (3). With these biosensor cells, the aggregation of tau induced by an exogenous tau seed can be quantified using FRET flow cytometry (15). FRET allows for the quantification of intracellular tau aggregates in the biosensor cells due to unique fluorescence emission patterns that occur when CFP and YFP fluorescence are in very close proximity within the aggregate. These emission patterns do not occur when tau is not aggregated.

Tau Strains

Nineteen different strains of tau (identified by morphologic characteristics) have been isolated from patient brains and recombinant fibrils and are propagated stably in separate HEK-293 cell lines in the Diamond laboratory (4). During this project, eleven cell lines (DS 1, 5, 6, 8, 9, 10, 13, 14, 15, 16, 17) were grown, harvested, and lysed. The lysate was used as source material for strain specific tau aggregates to induce seeding in biosensor cells.

Tau Strain Cell Lysate

Lysate was made using the following protocol: 1) previously frozen cells containing tau strain aggregates were thawed on ice; 2) a "TriX" buffer solution was made (PBS + Protease Inhibitor + 0.05% of Triton-X was combined and homogenized with a Fisher Scientific table top Vortex Mixer); 3) the cell pellet of one T300 flask (cells were highly confluent) was re-suspended in 1 ml of TriX buffer; 4) the solution was homogenized with a probe sonicator at 40% for 40 bursts (corresponding to ~80 watt, QSonica); 5) the solution was centrifuged at 16,000 x g for 30 minutes at room temperature; 6) the supernatant was collected; 7) the protein level of the supernatant was quantified using a Bradford Assay according to the manufacturers instructions; 8) the supernatant was frozen at -80°C until needed.

Heparinoids

Physiologic heparin is a long polysaccharide made of repeating disaccharide units (Figure 13) which varies in length but averages roughly 40 units. Various heparinoids have been identified with binding potential to tau (16). These include heparinoids of varying length (4, 8, 12, and 16 disaccharide units) and with distinct sulfate moieties removed from their sugar chains, including 2-O-desulfated (De-2-O), 6-O-desulfated (De-6-O), and N-desulfated (De-N) (see Figure 13). Heparinoids were purchased from amsbio (Cambridge, MA).

CRISPR/Cas9 Altered Cells

Genetic knockouts were performed using CRISPR/Cas9 as detailed by Stopschinski, et al (16). The following three genes, each important in HSPG formation as discussed in the introduction, were knocked out: *EXT1*, *NDST1*, *HS6ST2*.

Seeding Assay for Heparin/Heparinoid Inhibition

Day 1: First, HEK-293 P301S biosensor cells were plated in a 96-well plate at 20,000 cells/well. Second, cell lysate containing tau aggregates (15 ug total protein/well) was combined with heparin or heparinoids (conditions included: 0, 0.2, 0.66, 2, 6.6, 20, & 200 ug/mL) and cell culture media and was incubated for 24-hours at 4 °C.

Day 2: After 24-hours, the solution of cell lysate, heparin/heparinoids, and media was added to cultured cells so that 15 ug of total cell lysate protein was added to each well.

Day 4: After a 48-hour incubation period, cells were washed, dissociated from the tissue culture dish (using 0.05% trypsin), fixed in paraformaldehyde (2%), and then resuspended in flow cytometry buffer (HBSS + 1% FBS and 1 mM EDTA). Then, an LSRFortessa SORP (BD Biosciences) flow cytometer was used to measure FRET as described previously (3).

Seeding Assay with HSPG Knockout Cells

Day 1: First, three cell lines, each with a CRISPR/Cas9 gene knockout affecting a unique portion of the HSPG formation pathway (16), a scrambled control cell line, and HEK P301S biosensor cells were plated in a 96-well plate at 20,000 cells/well. Second, cell lysate containing tau aggregates (15 ug total protein/well) was combined with cell culture media and was incubated for 24-hours at 4 °C. Of note, knockout cells were treated with puromycin for selection two days prior to the experiment.

Day 2: After 24-hours, the solution of cell lysate and media was added to cultured cells so that 15 ug of total cell lysate protein was added to each well.

Day 4: After a 48-hour incubation period, cells were washed, dissociated from the tissue culture dish (using 0.05% trypsin), fixed in paraformaldehyde (2%), and then resuspended in flow cytometry buffer (HBSS + 1% FBS and 1 mM EDTA). Then, an LSRFortessa SORP (BD Biosciences) flow cytometer was used to measure FRET as described above.

Data Analysis

Flow cytometer data was analyzed using FlowJo version 10 software (Treestar Inc.). FRET was quantified as previously described by the laboratory (3, 15). Quantitative analysis, including dose response curves and IC50 calculations, was completed using GraphPad Prism version 7 software for Windows.

CHAPTER 3: Results

Tau Strain Heparin Sensitivity

Data collected from heparin dose titrations showed that sensitivity of heparin inhibition of uptake was similar between strains DS 9 and DS 10 (Figures 1 and 2). The average reduction in seeding at maximal heparin concentrations (200 ug/mL) was ~ 95% for both strains (Figure 1). The results of seven independent experiments showed a consistent pattern of nearly identical sensitivity between the two strains. This is evidenced by the high degree of similarity between the dose response curves (Figure 2) and IC_{50} concentrations (DS 9: 99 nM, DS 10: 105 nM) for both strains.

Heparin titrations were performed on 11 of the 19 tau strains identified in the Diamond laboratory (Figures 3 and 4) and showed that most strains have heparin sensitivity similar to DS 9 and DS 10. Seeding in these strains was also reduced by ~95% at maximal heparin concentrations with an IC_{50} near 100 nM (DS 5 = 107 nM; DS 6 = 78 nM; DS 8 = 50 nM; DS 13 = 51 nM; DS 14 = 97 nM; DS 15 = 72 nM; DS 16 = 131 nM; DS 17 = 69 nM). However, DS 5 and DS 6 were less sensitive to heparin than other strains (Figure 5). At maximal heparin concentrations (200 ug/mL), seeding in DS 5 and DS 6 was reduced by only 83% and 91%, respectively, noticeably lower than the 95-99% reduction in the other strains.

Tau Strain Seeding in HSPG Gene Knockout Cell Lines

Results from experiments testing tau aggregate uptake after CRISPR/Cas9 genetic knockout of important enzymes in the HSPG synthesis pathway are shown in Figure 6. This data shows a significant decrease in intracellular seeding for all strains in cells with the *EXT1* knockout (change in seeding: DS 5: -38%, DS 6: -50%, DS 9: -64%, DS 10: -67%, DS 15: -51%, DS 17: -65%) and the *NDST1* knockout (change in seeding: DS 5: -26%, DS 6: -38%, DS 9: -44%, DS 10: -50%, DS 15: -28%, DS 17: -45%) as compared to the scrambled control. The decrease observed in the *EXT1* knockout was larger than in the *NDST1* knockout. When comparing relative seeding reduction across strains in these knockout cells it was apparent that DS 5, DS 6, and DS 15 had less reduction than the other strains (-38%, -50%, and -51% respectively as compared to roughly -65% for other strains). This sensitivity was ordered similarly to that seen in the previous experiment (Figure 3 and 4) where a relatively higher level of residual seeding was observed at the highest heparin concentrations in these three strains (DS 5 > 6 >

15) as compared to the others.

The results for the *HS6ST2* knockout were different than those of the two previously mentioned knockouts. In these cells, intracellular seeding did not decrease, but rather increased substantially across all strains of tau tested (Figure 6). The change in seeding was as follows: DS 5: +49%, DS 6: +58%, DS 9: +28%, DS 10: +13%, DS 15: +27%, DS 17: +23%.

Tau Strain Heparinoid Sensitivity

We also tested DS 9 and DS 10 against various heparinoids to gain a better understanding of how size and sulfation patterns of heparin affect binding to tau to inhibit uptake into cells and whether these factors differ between strains (Figures 7-12).

We tested heparinoids of 4, 8, 12, and 16 disaccharide units (dp4, dp8, dp12, dp16) to evaluate the effect of varying length on the inhibition of tau uptake (Figures 7-10). Results showed that larger heparinoid molecules better inhibit seeding and that differences in size affect both strains similarly. The absolute reduction in seeding was as follows: for DS 9 (dp4 = 21%; dp8 = 27%; dp12 = 70%, dp16 = 63%), for DS 10 (dp4 = 19%; dp8 = 33%; dp12 = 64%, dp16 = 46%).

Three heparinoids with distinct sulfate moieties removed from their sugar chains were tested for ability to inhibit tau uptake (Figures 7, 8, 11, 12). These heparinoids included 2-O-desulfated (De-2-O), 6-O-desulfated (De-6-O), and N-desulfated (De-N) (see Figure 13). The results showed that N-sulfation is most important for inhibition of seeding, followed by 6-O-sulfation, followed by 2-O-sulfation. At the highest concentration tested (200 ug/mL), absolute seeding reduction when these moieties were removed was as follows: for DS-9 (De-2-O = 65%; De-6-O = 52%; De-N = 35%), for DS-10 (De-2-O = 53%; De-6-O = 25%; De-N = 13%). These results show a slight difference in magnitude of reduction between strains. However, the relative order of importance of the sulfate moieties for tau binding was shared between strains (N > 6-O > 2-O).

CHAPTER 4: Conclusions and Recommendations

Data from these experiments has shed light on the two important questions which motivated this investigation, providing evidence that: 1) most strains of tau seem to have similar sensitivity to heparin/heparinoid inhibition of uptake, but some show subtle differences, and 2) there seem to be clear size and sulfation patterns of HSPGs that are important for the uptake of tau.

Studies with Heparinoids

As previously mentioned, scientific data suggests that heparin mimetics may inhibit prion pathogenesis in animal models (13). In addition, the Diamond laboratory has demonstrated in previous publications that tau uptake is sensitive to heparin inhibition in cell models (2, 16). However, it was unknown if differences in heparin inhibition of uptake exist between tau strains. Our initial hypothesis was that tau strains would have different sensitivity to heparin based on their distinct structures. We considered this a plausible expectation given that strains with different conformations might have differential binding to heparan sulfate side chains.

The results of multiple heparin titrations showed highly similar responses to heparin in most strains (refuting our hypothesis) as well as subtle differences in a few strains (supporting our hypothesis). These are interesting findings for multiple reasons. First, it suggests that certain strains, though morphologically diverse, likely bind heparin (and thus HSPGs) in a similar manner. This provides evidence that heparin sensitivity cannot be reliably predicted by tau aggregate morphology visible by fluorescence microscopy. Second, it suggests that the cellular uptake of many strains can be inhibited by the same molecule, heparin. This is important information for the development of pharmacotherapy targeting tau aggregates in different tauopathies, suggesting the same heparinoid could be used across many strains. Third, the persistence of intracellular seeding in DS 5 and DS 6 at maximal heparin concentrations suggests these strains may have differential heparin binding compared to other strains. This provides evidence of a possible mechanistic explanation to support the hypothesis that different tau strains underlie different tauopathies (4).

Strong evidence has shown that tau uptake is mediated by cell surface HSPGs (2). To evaluate the relative importance of various size and sulfation patterns of HSPGs, heparinoid titrations were used to inhibit tau uptake into cells. Results from these experiments bolster the evidence that certain size and

sulfation patterns on heparin have specific importance for tau binding. The data also shows that these qualities are consistent between two strains and recombinant tau. Our experiments showed that larger heparinoids are better at inhibiting tau aggregates from entering cells (dp16 & dp12 > dp8 & dp4). We also showed that in terms of importance in tau binding affinity, N-sulfation is more important than 6-O-sulfation which is more important that 2-O-sulfation. Interestingly, other researchers have also found that 6-O sulfation is important for tau uptake (17-18). This pattern remained consistent between DS 9 and DS 10.

Studies with knockout cell lines

To further explore sulfation patterns of HSPGs important for tau uptake, cell lysate containing various tau strains was added to different polyclonal cell lines in which important enzymes in the HSPG formation pathway had been knocked out with CRISPR/Cas9. These enzymes support extension (*EXT1*), N-sulfation (*NDST1*), and 6-O-sulfation (*HS6ST2*) of the heparan sulfate side chains. Data from these experiments showed a lower relative reduction of seeding in DS 5 and DS 6 (as compared to other strains tested) in *EXT1* and *NDST1*. This pattern also held true for DS 15. Though the difference was subtle, this could be a clue that certain strains have differential binding to HSPGs. Alternatively, it may hint that certain strains can enter cells via a non-HSPG entry mechanism. This possibility is important to consider given its potential implications in our understanding of tau pathobiology and to our approach to designing and using strain specific therapeutics to halt the spread of tau pathology.

The results obtained from testing the *HS6ST2* knockout are difficult to interpret. On one hand, if the results are reproducible (and knockout of *HS6ST2* increases seeding induced by strains and decreases seeding induced by recombinant tau), this would be a very important finding which shows differential behavior of different tau aggregates. On the other hand, the results could be attributable to errors in the experimental system and not the actual biology. For example, we know the cells survived a puromycin treatment, providing evidence that the knockout was functional, but it is still possible that the gene alteration was not present in the cells. Additionally, the cells were thawed (not freshly made) and this could have adversely affected their quality. Also, the limited number of independent experiments makes the result less reliable. In future experiments, confirmation of genetic knockouts should be done using a Western Blot or by making a monoclonal cell line and sequencing to confirm the knockout of the target gene. Also, a recombinant tau control should be used to allow comparison to

previous experiments.

Conclusions

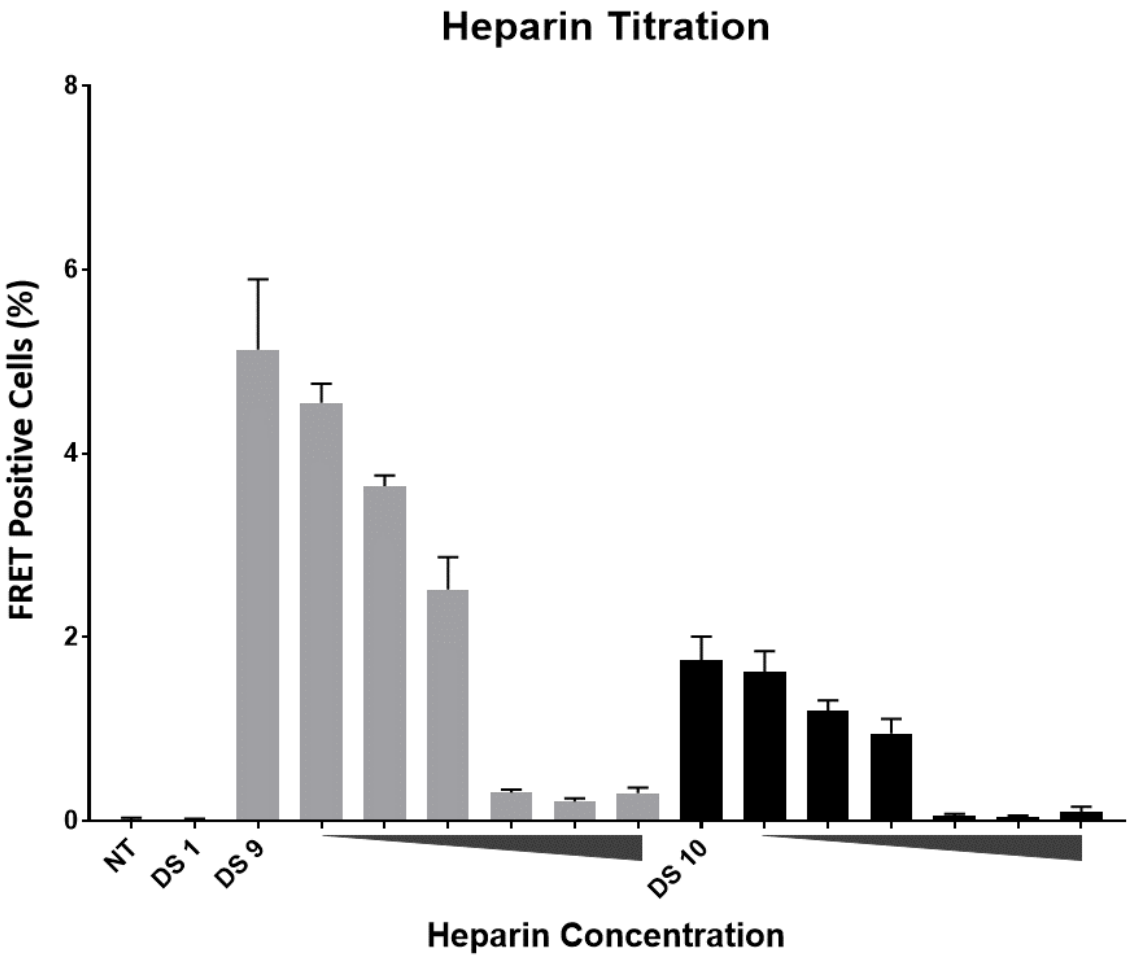
This project has served to further our understanding of tau protein pathobiology. Our data suggests that there are similarities in cellular uptake between many strains of tau, but that a one-size-fits-all approach to understanding uptake may not be sufficient. Some strains may exhibit distinct behavior. If this proves to be true, strain specific diagnostics and therapy may be required to target the exact strain or strains that affect a patient. Additionally, data from this project has added to our understanding of HSPG size and sulfation patterns required for tau uptake, improving our understanding of tau physiology. These results match other data gathered in the laboratory using genetic knockout cell lines and recombinant tau which were published recently in the Journal of Biological Chemistry (16). In conclusion, this project furthers our understanding of the interactions between tau and HSPGs and improves our understanding of strains in tauopathies. It can also contribute to novel approaches to tauopathy pharmacotherapy by helping inform the design of small molecule inhibitors to directly block the tau-HSPG interaction.

Future Directions

Future directions for this work include testing additional strains of tau for heparin sensitivity to determine if there are other strains which are differentially inhibited. It will also be important to test additional strains of tau for heparinoid sensitivity to further explore strain specific binding based on size and sulfation of HSPGs. Additional follow-up work should be done with regards to the result obtained for the *HS6ST2* knockout. If important differences exist between tau strains and recombinant tau in the sulfation pattern required for uptake, this will need to be understood to improve our understanding of tau pathobiology, our model experimental systems, and our approach to pharmacotherapy. The laboratory plans to conduct these experiments as well as additional seeding experiments with knockout cells using Alzheimer's Disease (AD) brain lysate as seeding material. It will be fascinating to see if tau aggregates from AD brain samples behave similarly to the strains tested here.

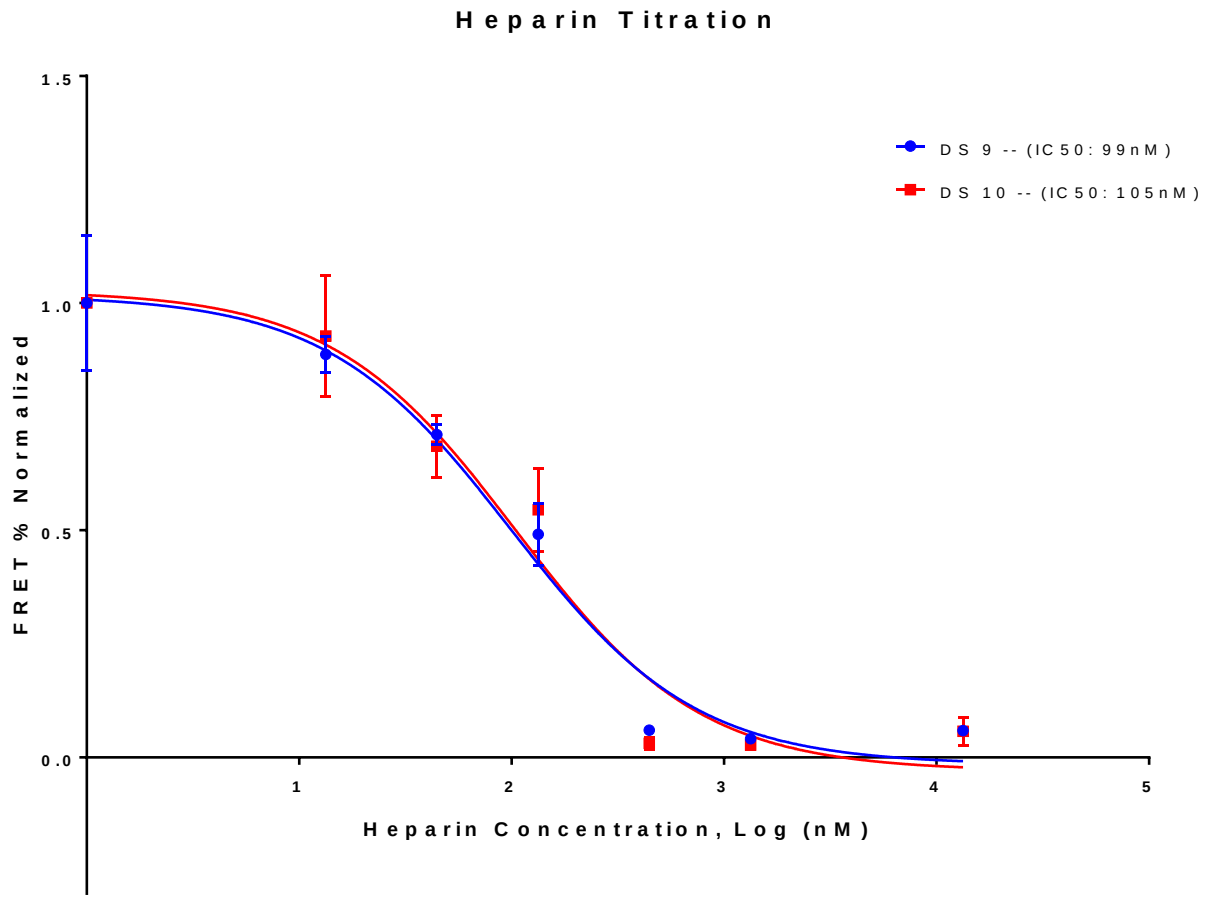
LIST OF FIGURES

Figure 1:



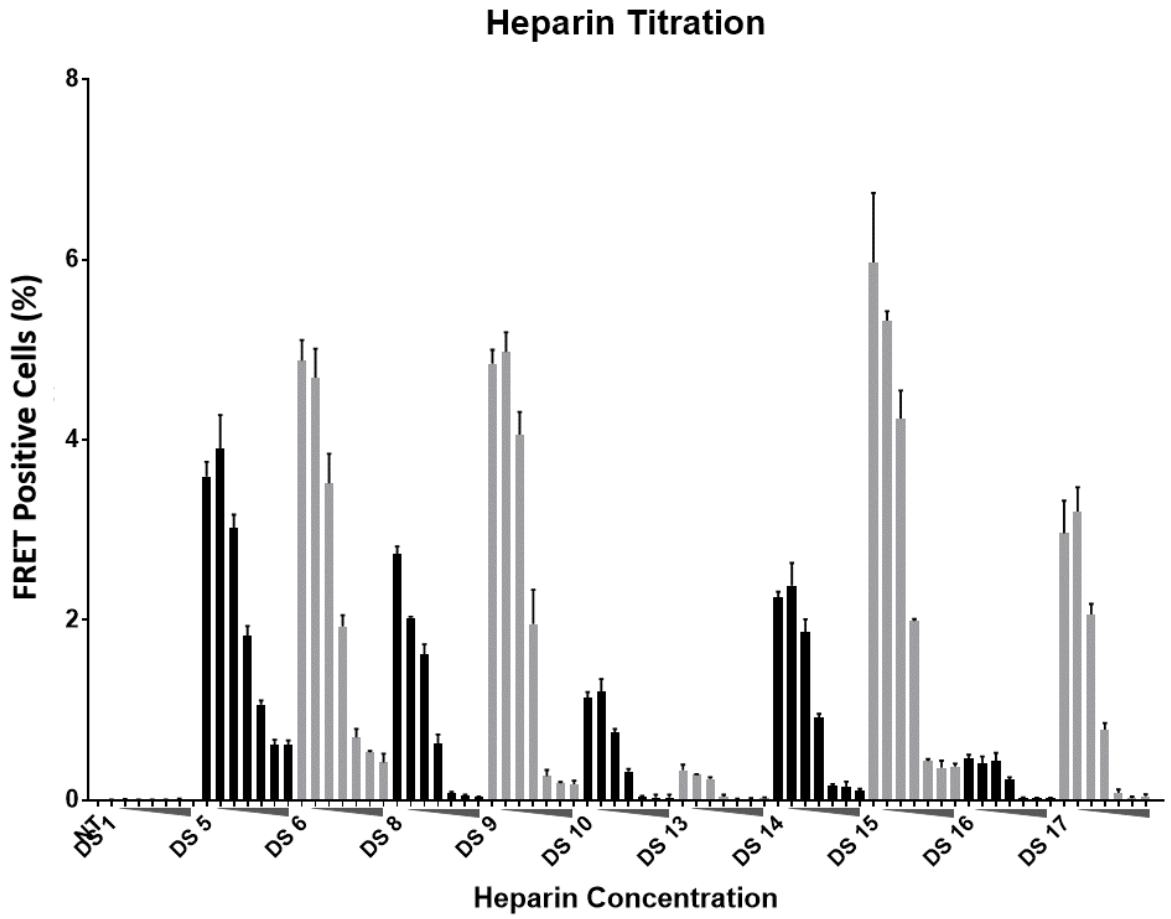
Tau strains DS 9 and DS 10 were incubated with increasing concentrations of heparin (0, 0.2, 0.66, 2, 6.66, 20, 200 ug/mL from left to right) before addition to cultured cells. Though DS 9 induced a higher absolute amount of seeding, both strains showed similar inhibition patterns to increasing heparin concentrations. Absolute seeding reduction at the highest heparin concentrations was ~95% for both strains. Seven independent experiments showed a similar pattern.

Figure 2:



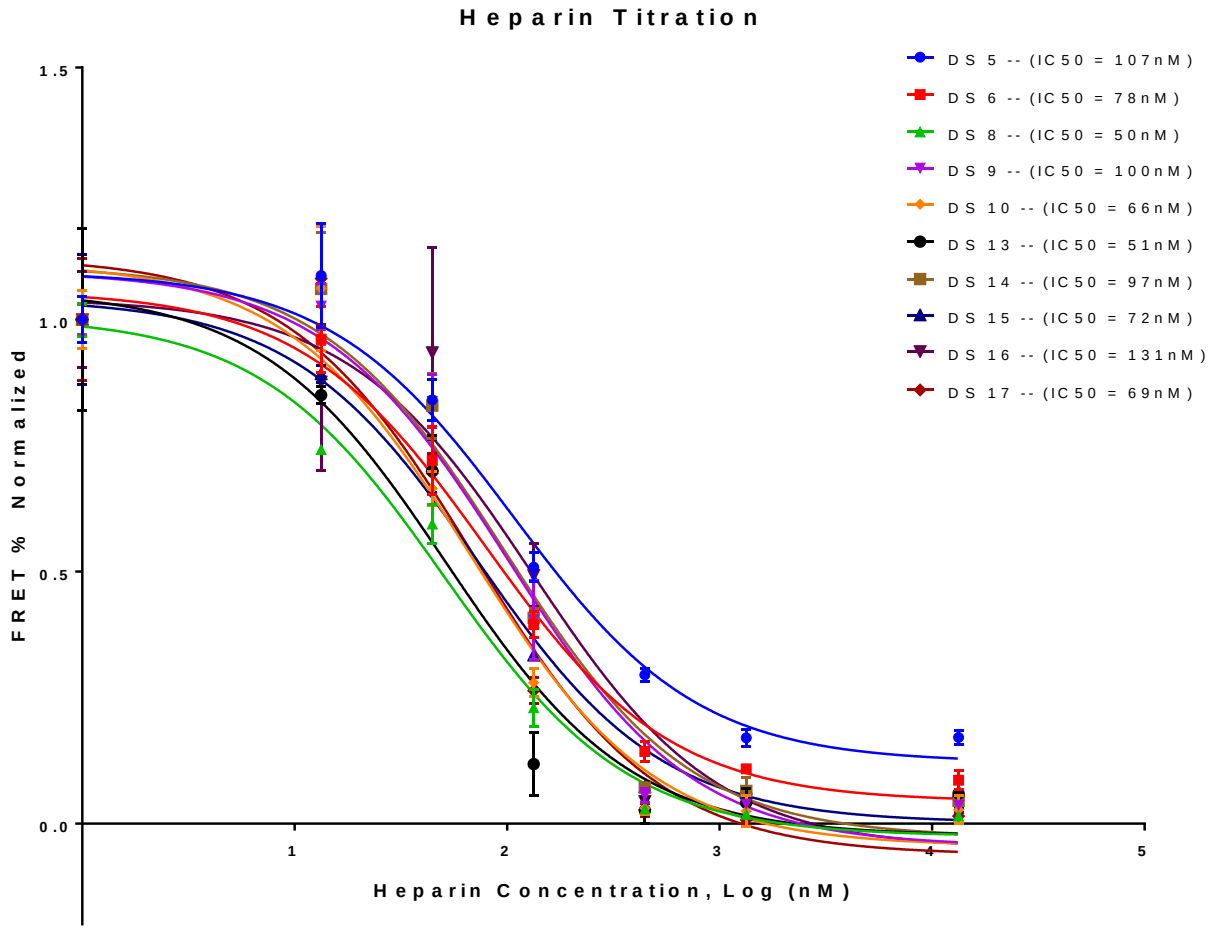
The data from Figure 1 is represented here as dose response curves for DS 9 and DS 10 (normalized to 1.0). This figure highlights the similarity of the heparin inhibition of both strains. The IC50 concentrations are almost identical between strains (DS 9 = 99 nM & DS 10 = 105 nM).

Figure 3:



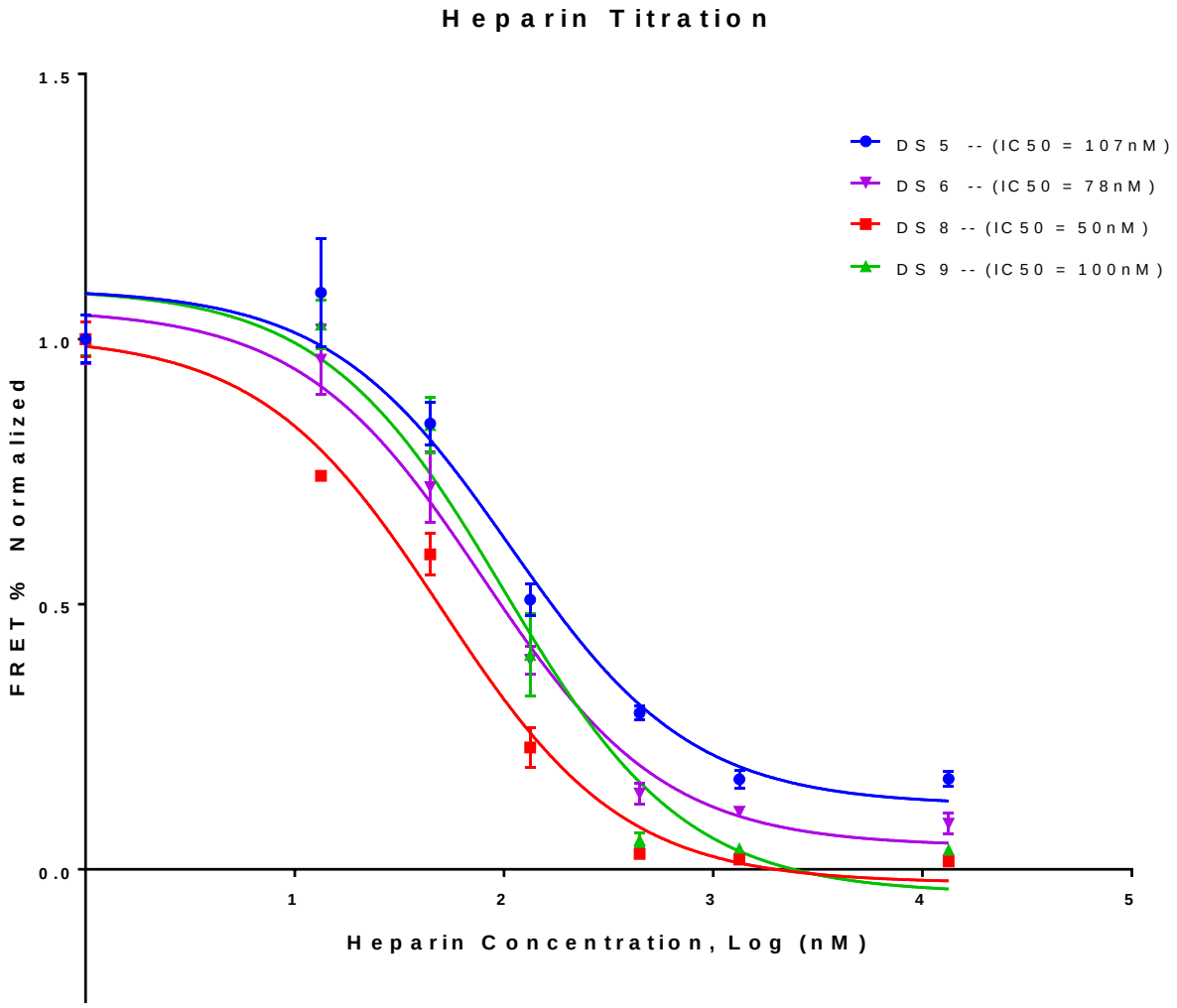
Tau strains were incubated with increasing concentrations of heparin (0, 0.2, 0.66, 2, 6.66, 20, 200 ug/mL from left to right) before addition to cultured cells. Unique strains induced varying amounts of seeding, but all the strains tested were highly sensitive to increasing concentrations of heparin. Of note, DS 5 and DS 6 (to a lesser extent) show higher relative amounts of seeding at maximal heparin concentrations when compared to the other strains. Most strains show a maximal reduction 95-99%, whereas maximal reduction for DS 5 = 83% and DS 6 = 91%.

Figure 4:



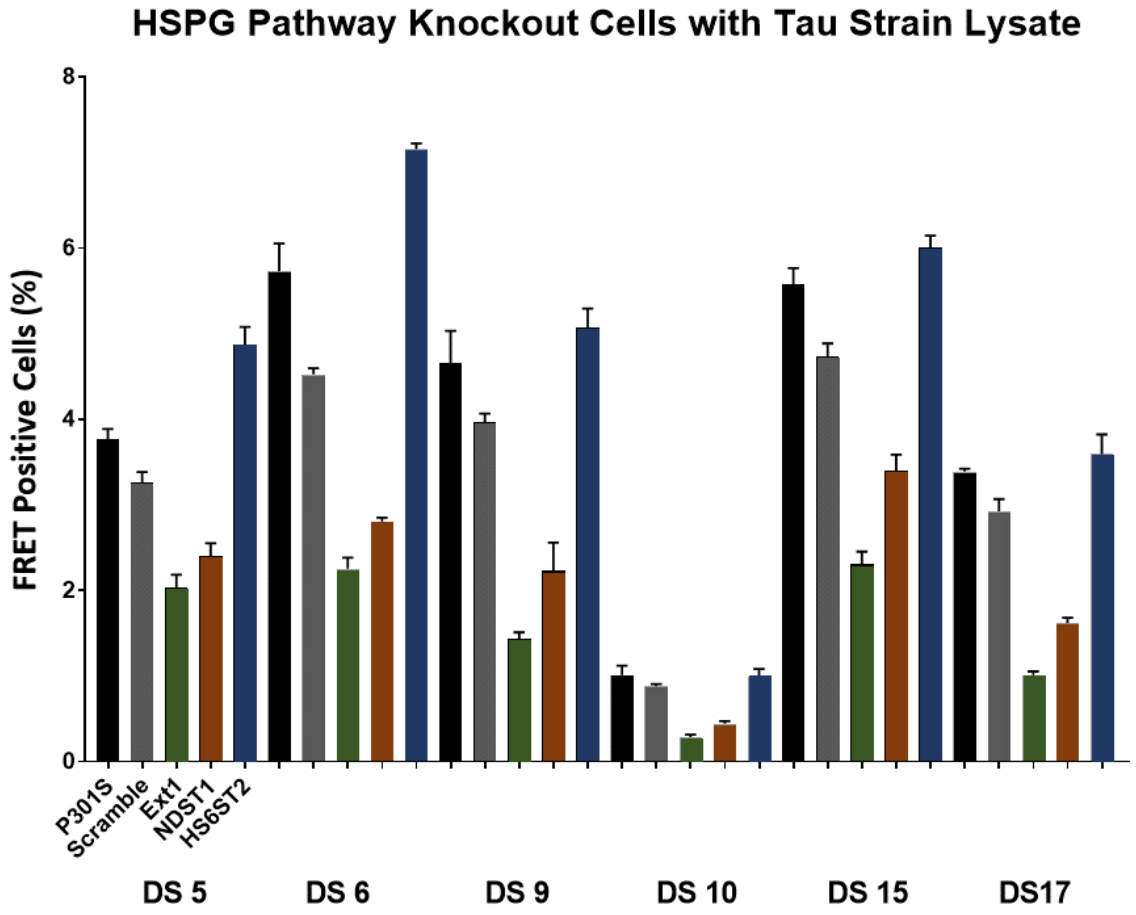
The data from Figure 3 is represented here as normalized dose response curves, highlighting that multiple tau strains show an overall similar pattern of heparin inhibition. The IC50 concentrations for these strains fall within a relatively narrow range from 50 nM to 131 nM. All of these strains were highly heparin sensitive, but there are some subtle differences. For example, seeding for DS 5 and DS 6 remains higher than other strains at maximal heparin concentrations of 200 ug/mL.

Figure 5:



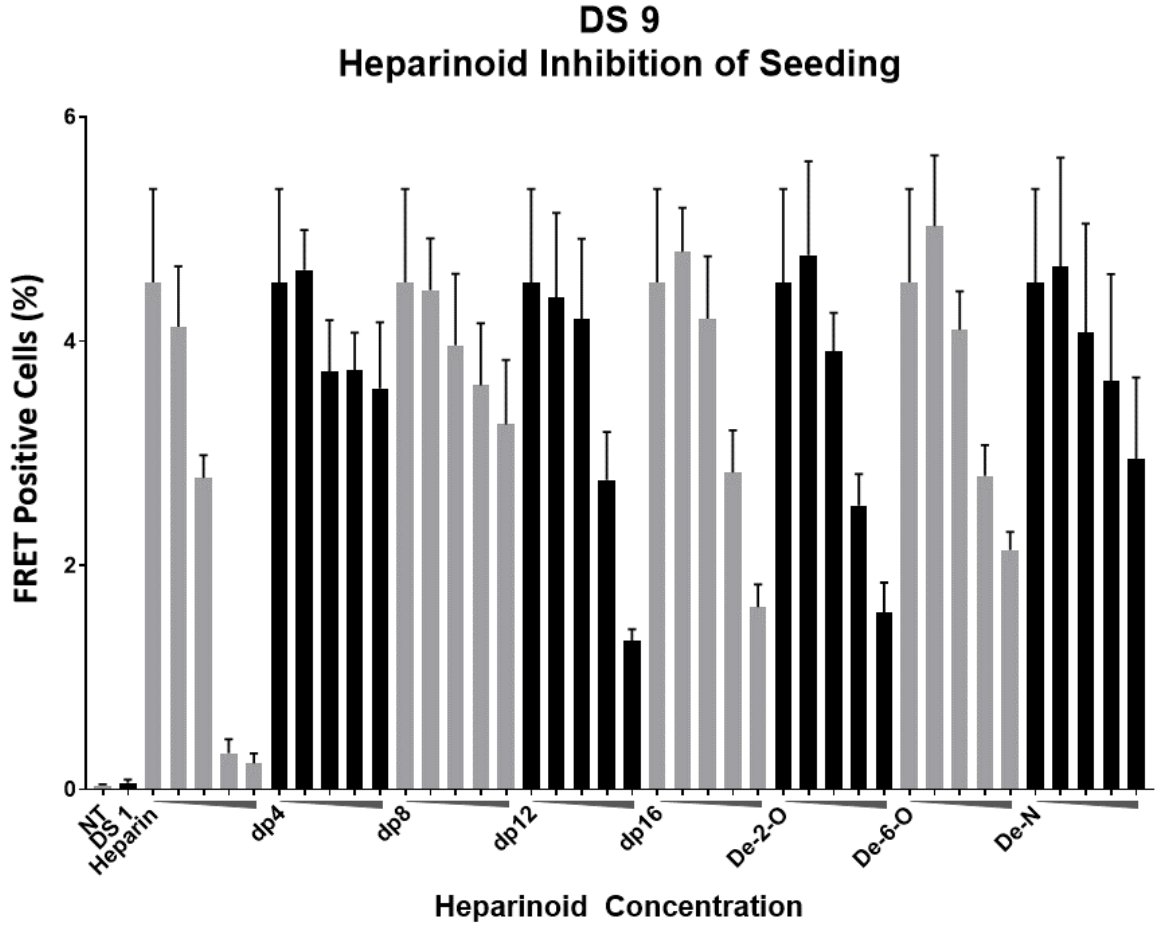
These four strains have been graphed separately from the others in Figure 4 to highlight the subtle differences that were observed between strains. DS 9 is shown as a reference to represent the average sensitivity of strains. When compared to DS 9, DS 5 and DS 6 appear less heparin sensitive and DS 8 appears more sensitive. One can see from these curves that a significant amount of seeding remains for DS 5 and DS 6 even at the highest heparin concentrations of 200 ug/mL (17% and 9%, respectively). In the other strains seeding was reduced almost to baseline at these high heparin concentrations.

Figure 6:



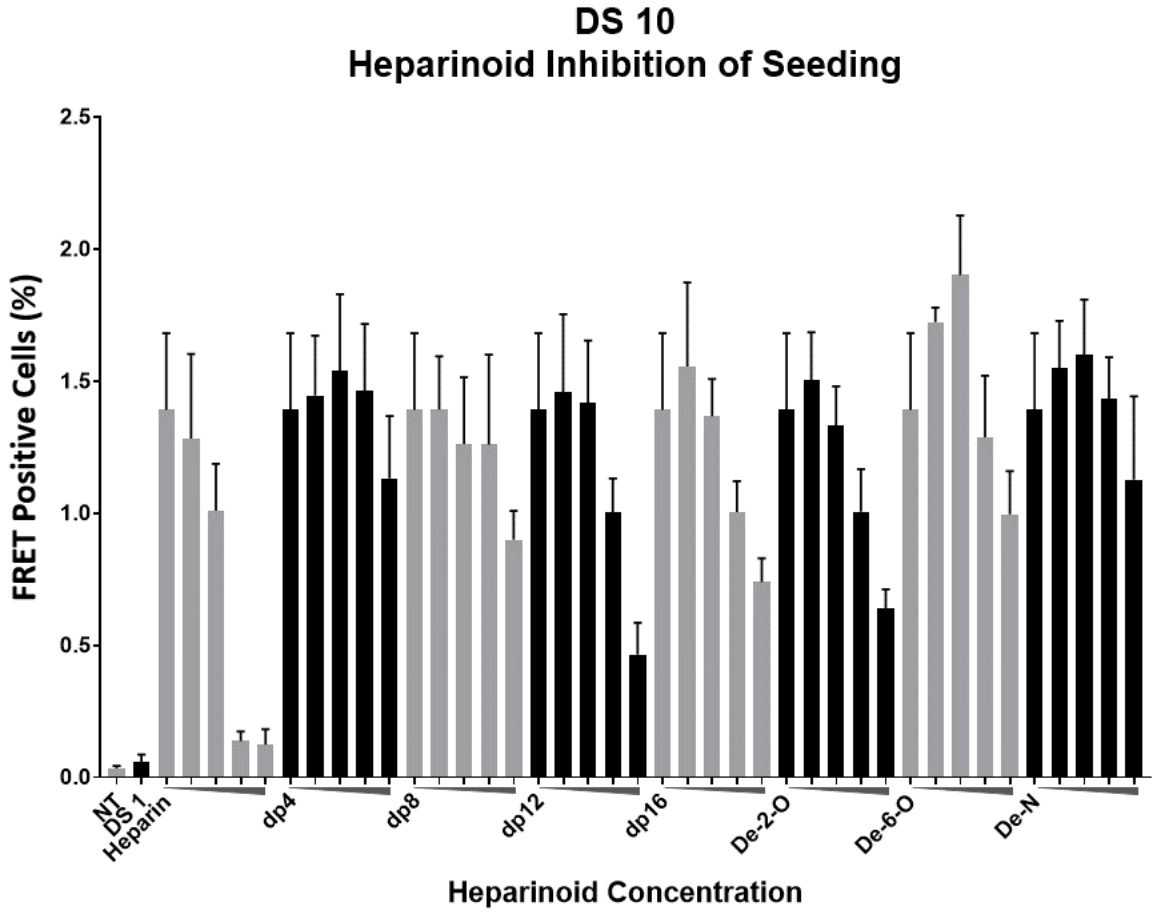
Biosensor cells with various genetic knockouts in the HSPG synthesis pathway were treated with cell lysate harboring different tau strains. Results showed a significant decrease in intracellular seeding for all strains in cells with the *EXT1* knockout (change in seeding: DS 5: -38%, DS 6: -50%, DS 9: -64%, DS 10: -67%, DS 15: -51%, DS 17: -65%) and the *NDST1* knockout (change in seeding: DS 5: -26%, DS 6: -38%, DS 9: -44%, DS 10: -50%, DS 15: -28%, DS 17: -45%) as compared to the scrambled control. When comparing relative seeding reduction across strains it was apparent that DS 5, 6, and 15 had significantly less reduction than the other strains (-38%, -50%, and -51% respectively as compared to roughly -65% for other strains in the *EXT1* knockout). The results in the *HS6ST2* knockout were surprising. In these cells, intracellular seeding did not decrease, but rather increased substantially across all strains of tau tested (change in seeding: DS 5: +49%, DS 6: +58%, DS 9: +28%, DS 10: +13%, DS 15: +27%, DS 17: +23%). This was unexpected as other experiments in the laboratory have shown a decrease in recombinant tau seeding in cells lines with this knockout.

Figure 7:



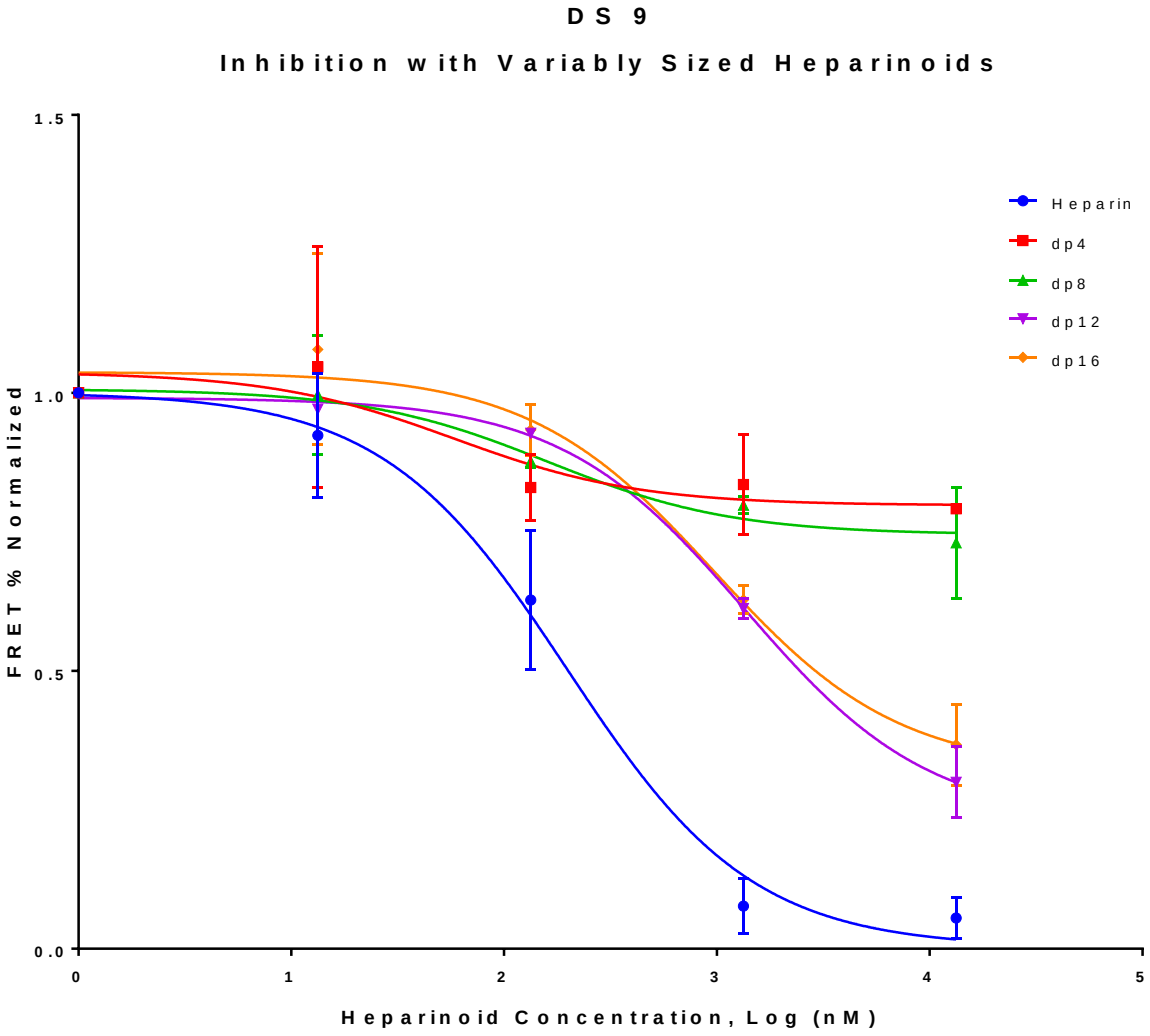
Tau strain DS 9 was incubated with increasing concentrations of heparin (0, 0.2, 2, 20, 200 ug/mL from left to right) before addition to cultured cells. Seeding was reduced by varying degrees based on the specific heparinoid used for inhibition. Heparinoids labeled dp4, dp8, dp12, and dp16 are heparinoids of 4, 8, 12, and 16 disaccharide units. Heparinoids labeled De-2-O, De-6-O, and De-N are heparinoids desulfated at the 2-O, 6-O, and N positions. The most effective inhibiting agent was heparin. Absolute reductions in seeding for other heparinoids are as follows: dp4 = 21%; dp8 = 27%; dp12 = 70%; dp16 = 63%; De-2-O = 65%; De-6-O = 52%; De-N = 35%. This data represents the results of two independent experiments and matches other data obtained in the laboratory using recombinant tau and genetic knockouts of enzymes in the HSPG synthesis pathway.

Figure 8:



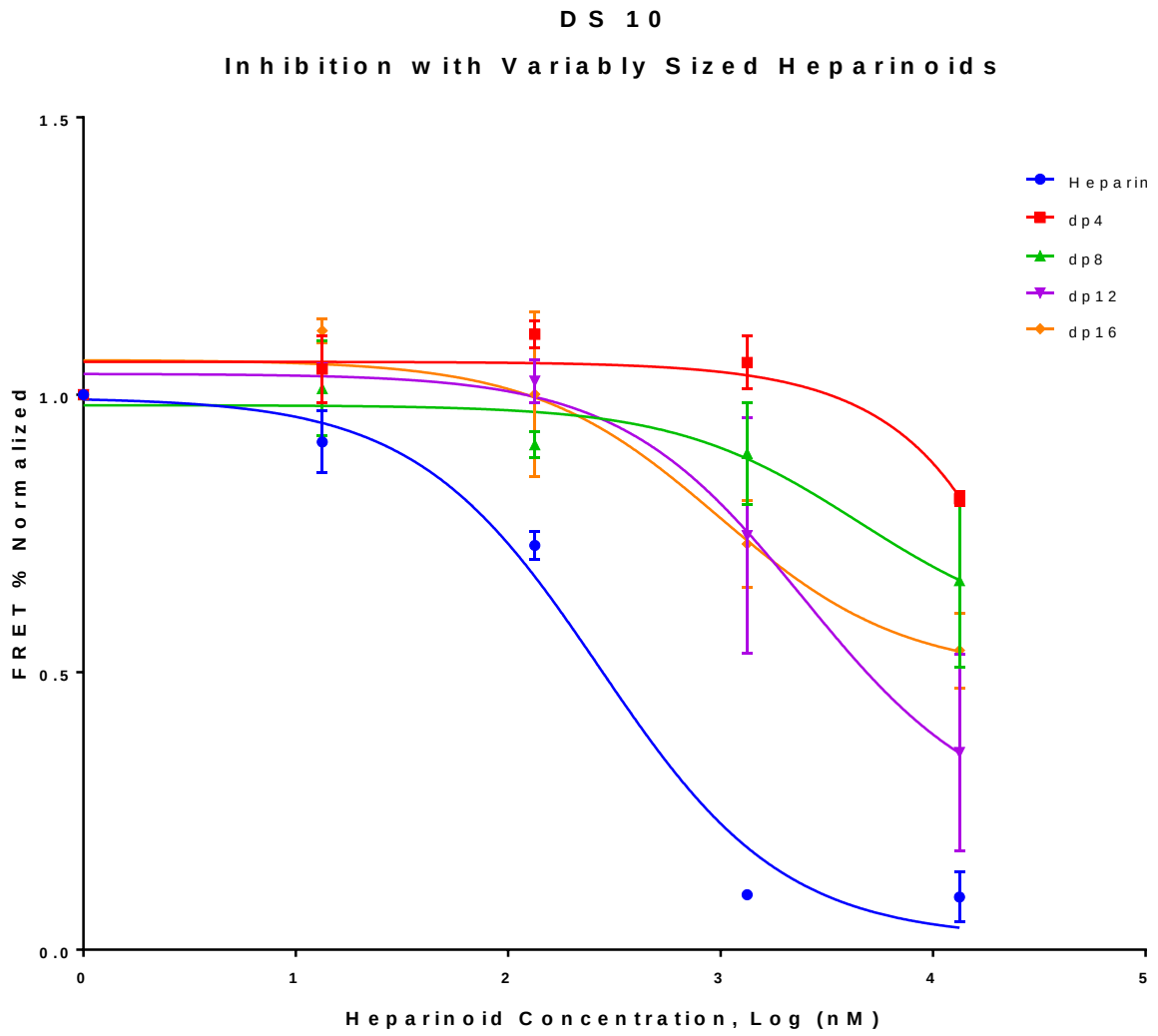
Tau strain DS 10 was incubated with increasing concentrations of heparin (0, 0.2, 2, 20, 200 ug/mL from left to right) before addition to cultured cells. Seeding was reduced by varying degrees based on the specific heparinoid used for inhibition. Heparinoids labeled dp4, dp8, dp12, and dp16 are heparinoids of 4, 8, 12, and 16 disaccharide units. Heparinoids labeled De-2-O, De-6-O, and De-N are heparinoids desulfated at the 2-O, 6-O, and N positions. The most effective inhibiting agent was heparin. Absolute reductions in seeding for other heparinoids are as follows: dp4 = 19%; dp8 = 33%; dp12 = 64%; dp16 = 46%; De-2-O = 53%; De-6-O = 25%; De-N = 13%. This data represents the results of two independent experiments and matches other data obtained in the laboratory using recombinant tau and genetic knockouts of enzymes in the HSPG synthesis pathway.

Figure 9:



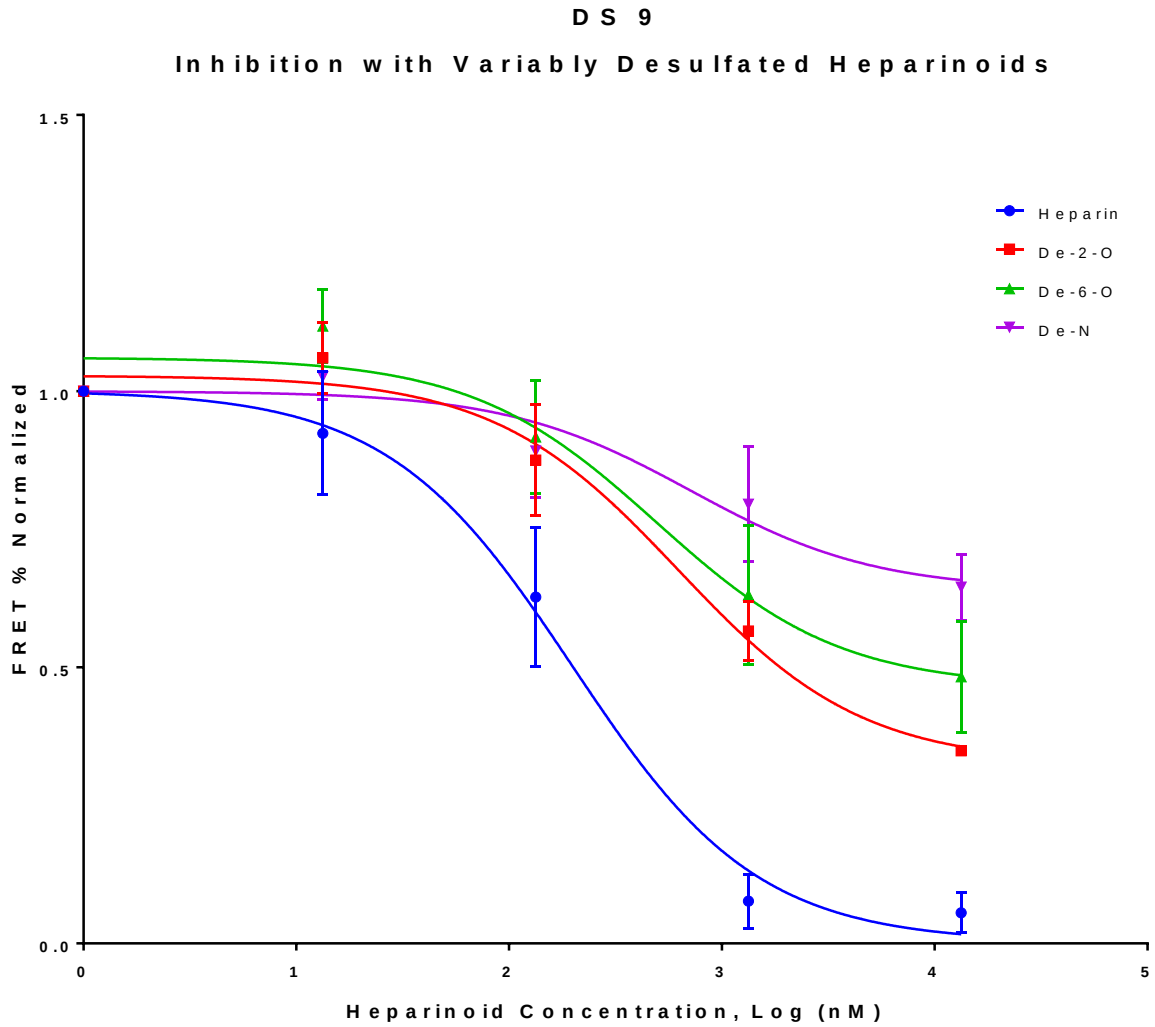
Data shown in Figure 7 is represented here as normalized dose response curves for DS 9. These curves illustrate the varying patterns of inhibition based on differing heparinoid modifications. Note the relative inhibition based on size. Larger heparinoids are more effective at reducing seeding (dp16 & dp12 > dp8 & dp4).

Figure 10:



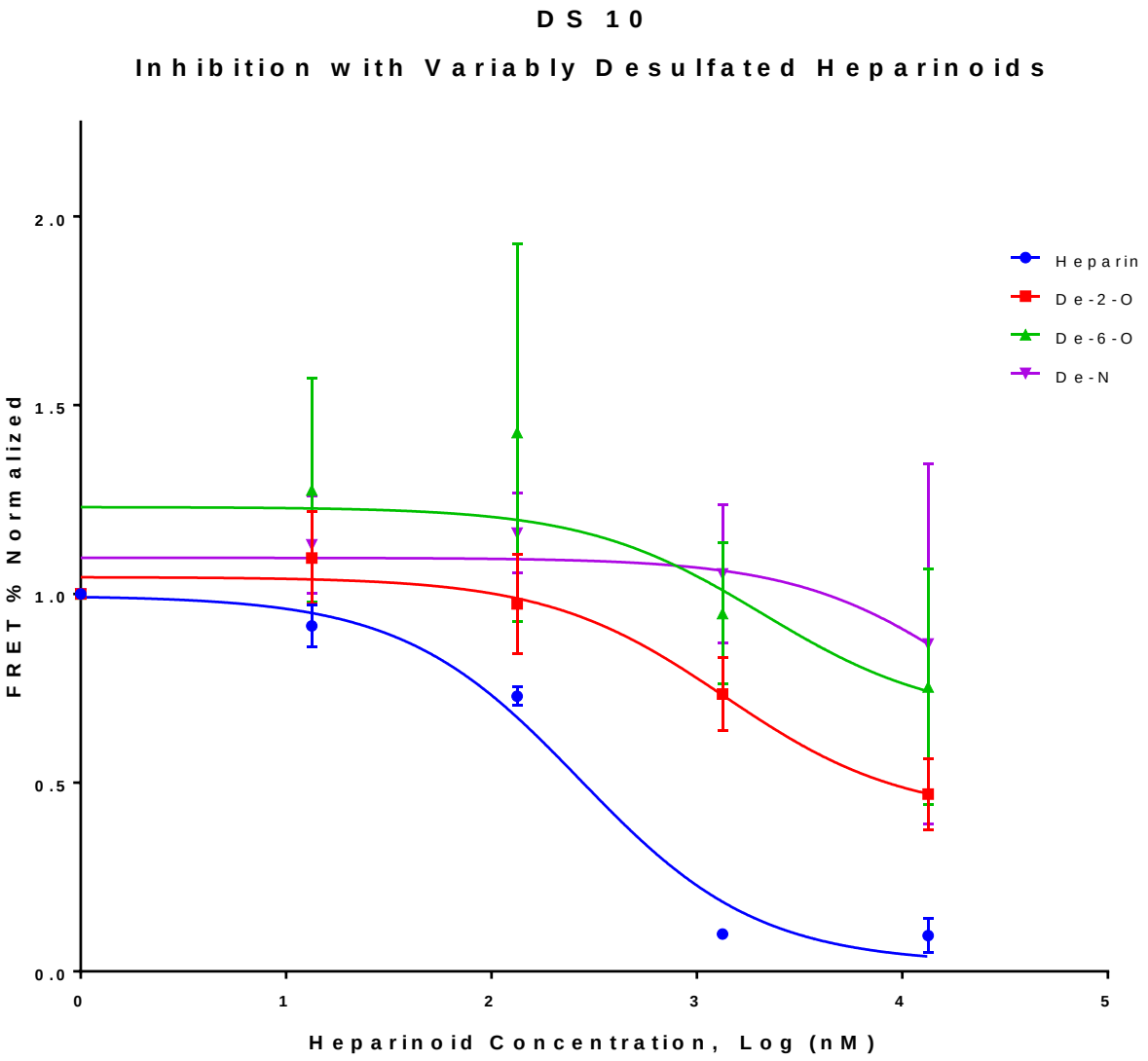
Data shown in Figure 8 is represented here as normalized dose response curves for DS 10. These curves illustrate the varying patterns of inhibition based on differing heparinoid modifications. Note the relative inhibition based on size. Larger heparinoids are more effective at reducing seeding (dp16 & dp12 > dp8 & dp4).

Figure 11:



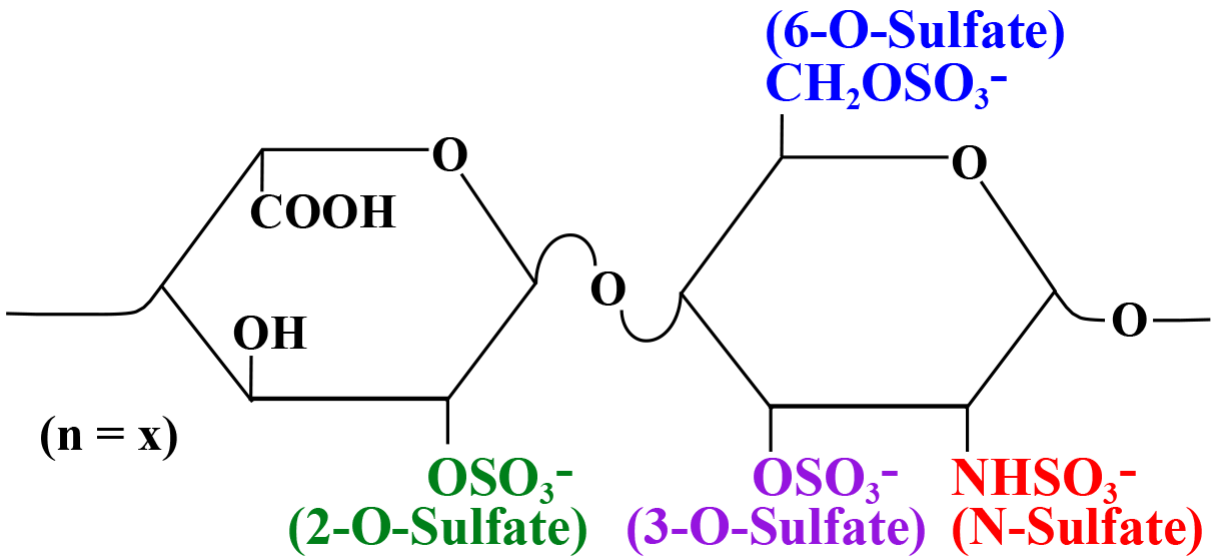
Data shown in Figure 7 is represented here as normalized dose response curves for DS 9. These curves illustrate the varying patterns of inhibition based on differing heparinoid modifications. Note the varying sulfate moieties, the removal of which affects seeding inhibition. The moieties most important for inhibition of tau seeding are N-sulfation > 6-O sulfation > 2-O sulfation.

Figure 12:



Data shown in Figure 8 is represented here as normalized dose response curves for DS 10. These curves illustrate the varying patterns of inhibition based on differing heparinoid modifications. Note the varying sulfate moieties, the removal of which affects seeding inhibition. The moieties most important for inhibition of tau seeding are N-sulfation > 6-O sulfation > 2-O sulfation.

Figure 13:



This cartoon depicts the structure of a heparin disaccharide unit. Heparin is composed of roughly 40 such units. Heparan sulfate is composed of 80-100 such units. The heparinoids used in the titrations in this project consisted of modifications to these sulfate moieties as well as modifications to the number of disaccharide units. (Image created by Dr. Brandon Holmes).

REFERENCES

1. Frost B, Jacks RL, Diamond MI. Propagation of tau misfolding from the outside to the inside of a cell. Journal of Biological Chemistry. 2009 May 8;284(19):12845-52.
2. Holmes BB, DeVos SL, Kfoury N, Li M, Jacks R, Yanamandra K, Ouidja MO, Brodsky FM, Marasa J, Bagchi DP, Kotzbauer PT. Heparan sulfate proteoglycans mediate internalization and propagation of specific proteopathic seeds. Proceedings of the National Academy of Sciences. 2013 Aug 13;110(33):E3138-47.
3. Holmes BB, Furman JL, Mahan TE, Yamasaki TR, Mirbaha H, Eades WC, Belaygorod L, Cairns NJ, Holtzman DM, Diamond MI. Proteopathic tau seeding predicts tauopathy in vivo. Proceedings of the National Academy of Sciences. 2014 Oct 14;111(41):E4376-85.
4. Sanders DW, Kaufman SK, DeVos SL, Sharma AM, Mirbaha H, Li A, Barker SJ, Foley AC, Thorpe JR, Serpell LC, Miller TM. Distinct tau prion strains propagate in cells and mice and define different tauopathies. Neuron. 2014 Jun 18;82(6):1271-88.
5. Sanders DW, Kaufman SK, Holmes BB, Diamond MI. Prions and protein assemblies that convey biological information in health and disease. Neuron. 2016 Feb 3;89(3):433-48.
6. Lee VM, Goedert M, Trojanowski JQ. Neurodegenerative tauopathies. Annual review of neuroscience. 2001 Mar;24(1):1121-59.
7. Hutton M, Lendon CL, Rizzu P, Baker M, Froelich S, Houlden H, Pickering-Brown S, Chakraverty S, Isaacs A, Grover A, Hackett J. Association of missense and 5′-splice-site mutations in tau with the inherited dementia FTDP-17. Nature. 1998 Jun;393(6686):702.
8. Poorkaj P, Bird TD, Wijsman E, Nemens E, Garruto RM, Anderson L, Andreadis A, Wiederholt WC, Raskind M, Schellenberg GD. Tau is a candidate gene for chromosome 17 frontotemporal dementia. Annals of neurology. 1998 Jun;43(6):815-25.
9. Spillantini MG, Murrell JR, Goedert M, Farlow MR, Klug A, Ghetti B. Mutation in the tau gene in familial multiple system tauopathy with presenile dementia. Proceedings of the National Academy of Sciences. 1998 Jun 23;95(13):7737-41.
10. Braak H, Braak E. Neuropathological stageing of Alzheimer-related changes. Acta neuropathologica. 1991 Sep 1;82(4):239-59.
11. Braak H, Braak E. Frequency of stages of Alzheimer-related lesions in different age categories. Neurobiology of aging. 1997 Jul 1;18(4):351-7.
12. Delacourte A, David JP, Sergeant N, Buee L, Wattez A, Vermersch P, Ghozali F, Fallet-Bianco C, Pasquier F, Lebert F, Petit H. The biochemical pathway of neurofibrillary degeneration in aging and Alzheimer's disease. Neurology. 1999 Apr 1;52(6):1158-.
13. Doh-Ura K, Ishikawa K, Murakami-Kubo I, Sasaki K, Mohri S, Race R, Iwaki T. Treatment of transmissible spongiform encephalopathy by intraventricular drug infusion in animal models. Journal of virology. 2004 May 15;78(10):4999-5006.
14. Holtzman DM, Morris JC, Goate AM. Alzheimer's disease: the challenge of the second century. Science translational medicine. 2011 Apr 6;3(77):77sr1-.
15. Furman JL, Holmes BB, Diamond MI. Sensitive detection of proteopathic seeding activity with FRET flow cytometry. Journal of visualized experiments: JoVE. 2015(106).
16. Stopschinski BE, Holmes BB, Miller GM, Manon VA, Vaquer-Alicea J, Prueitt WL, Hsieh-Wilson LC, Diamond MI. Specific glycosaminoglycan chain length and sulfation patterns are required for cell uptake of tau vs. α-synuclein and β-amyloid aggregates. Journal

of Biological Chemistry. 2018 May 11:jbc-RA117.
17. Zhao J, Huvent I, Lippens G, Eliezer D, Zhang A, Li Q, Tessier P, Linhardt RJ, Zhang F, Wang C. Glycan determinants of heparin-tau interaction. Biophysical journal. 2017 Mar 14;112(5):921-32.
18. Rauch JN, Chen JJ, Sorum AW, Miller GM, Sharf T, See SK, Hsieh-Wilson LC, Kampmann M, Kosik KS. Tau Internalization is Regulated by 6-O Sulfation on Heparan Sulfate Proteoglycans (HSPGs). Scientific reports. 2018 Apr 23;8(1):6382.

VITAE

William Prueitt is a 4th year medical student at the University of Texas Southwestern Medical School in Dallas, Texas. He grew up in Tulsa, Oklahoma and completed his undergraduate studies at the University of Oklahoma. Upon graduation from UT Southwestern he plans to begin residency training in psychiatry.

Permanent Address:
6343 Bordeaux Ave
Dallas, TX 75209