A synthetic heparinoid blocks Tau aggregate cell uptake and amplification

Received for publication, July 28, 2019, and in revised form, January 21, 2020. Published, Papers in Press, January 23, 2020, DOI 10.1074/jbc.RA119.010353

Barbara E. Stopschinski†, Talitha L. Thomas‡, Sourena Nadji†, Eric Darvish†, Linfeng Fan†, Brandon B. Holmes‡‡, Anuja R. Modi†, Jordan G. Finnell‡, Omar M. Kasher‡, Sandi Estill-Terpack‡, Hilda Mirbaha§, Hung S. Luu§, and Marc I. Diamond‡

From the †Center for Alzheimer’s and Neurodegenerative Diseases, Peter O’Donnell Jr. Brain Institute, and the ‡Department of Pathology, Children’s Health, University of Texas Southwestern Medical Center, Dallas, Texas 75390, the Department of Neurology, RWTH University Aachen, 52074 Aachen, Germany, ‡PharRaen Discovery LLC, Berkeley, Missouri 63134-3115, §Shanghai Acana Pharmtech Co. Ltd., Berkeley, Missouri 63134-3115, and the ‡‡Department of Neurology, University of California, San Francisco, California 94143

Edited by Paul E. Fraser

Tau aggregation underlies neurodegeneration in Alzheimer’s disease and related tauopathies. We and others have proposed that transcellular propagation of pathology is mediated by Tau prions, which are ordered protein assemblies that faithfully replicate in vivo and cause specific biological effects. The prion model predicts the release of aggregates from a first-order cell and subsequent uptake into a second-order cell. The assemblies then serve as templates for their own replication, a process termed “seeding.” We have previously observed that heparan sulfate proteoglycans on the cell surface mediate the cellular uptake of Tau aggregates. This interaction is blocked by heparin, a sulfated glycosaminoglycan. Indeed, heparin-like molecules, or heparinoids, have previously been proposed as a treatment for PrP prion disorders. However, heparin is not ideal for managing chronic neurodegeneration, because it is difficult to synthesize in defined sizes, may have poor brain penetration because of its negative charge, and is a powerful anticoagulant. Therefore, we sought to generate an oligosaccharide that would bind Tau and block its cellular uptake and seeding, without exhibiting anticoagulation activity. We created a compound, SN7–13, from pentasaccharide units and tested it in a range of assays that measured direct binding of Tau to glycosaminoglycans and inhibition of Tau uptake and seeding in cells. SN7–13 does not inhibit coagulation, binds Tau with low nanomolar affinity, and inhibits cellular Tau aggregate propagation similarly to standard porcine heparin. This synthetic heparinoid could facilitate the development of agents to treat tauopathy.

This work was supported by RWTH University Aachen, Faculty of Medicine, Germany, through the Rotation Program for Junior Researchers (to B. E. S.), the Carl and Florence E. King Foundation (to B. E. S.), the Rainwater Charitable Foundation (to M. I. D.), and the Cure Alzheimer’s Fund (to M. I. D.). The authors declare that they have no conflicts of interest with the contents of this article.

This article contains supporting text and Figs. S1 and S2.

© 2020 Stopschinski et al. Published under exclusive license by The American Society for Biochemistry and Molecular Biology, Inc.

**The abbreviations used are:** HSPG, heparan sulfate proteoglycan; HS, heparan sulfate; GAG, glycosaminoglycan; LMWH, low-molecular-weight heparin; AD, Alzheimer’s disease; PT, prothrombin time; PTT, partial thromboplastin time; BLI, biolayer interferometry; MFI, median fluorescence intensity; RD, repeat domain; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; HBSS, Hanks’ balanced salt solution; FBS, fetal bovine serum; SEC, size-exclusion chromatography.
uptake of prion protein aggregates is mediated by HSPGs and can be blocked by GAG derivatives in vitro (9, 14, 15), and GAGs have been observed to reduce prion pathology in vivo (16, 17). Finally, low-molecular-weight heparins (LMWHs) such as neuroparin and enoxaparin have demonstrated neuroprotective effects in mouse models of Alzheimer’s disease (AD) (18–22). Taken together, GAGs may be a promising therapeutic target to inhibit the progression of pathology in tauopathies and other neurodegenerative disorders.

Because of its high molecular mass and charge, anticoagulation properties, and the associated risk of hemorrhage, heparin is not suitable for chronic administration to treat neurodegeneration. We thus sought to generate a low-molecular-weight heparinoid that potently inhibits Tau uptake and intracellular seeding and lacks anticoagulation activity.

Results

Synthesis of a heparin derivative with low molecular weight

We used a pentasaccharide analog of fondaparinux, a low-molecular-weight anticoagulant, as the starting molecule for the synthesis of SN7–13 (Fig. 1). Fondaparinux binds antithrombin (10, 11, 23). Three N-sulfate moieties in fondaparinux play a critical role, because their deletion significantly reduces its anticoagulation properties (24, 25). We previously found that N-sulfation of GAGs mediates binding to Tau and is required for its cellular uptake (9). We found that replacing N-sulfate moieties with “linkable linkers” retained Tau binding and inhibitory activities similar to heparin (Figs. 1 and 2). We linked several pentasaccharides by carbon chains (Figs. 1 and 2), thereby extending the GAG length without increasing the overall charge of the molecule. We generated and tested seven series of compounds. The final product was SN7–13 (Figs. 1 and 2), a polydisperse compound composed of 2–4 pentasaccharides with a molecular mass from 2.5 to 8 kDa. This molecular mass is significantly lower than the average molecular mass of standard heparin, which ranges from 3 to 30 kDa (26, 27).

SN7–13 does not affect the coagulation cascade

We first determined the coagulation properties of SN7–13 compared with heparin (Table 1). Like heparin, SN7–13 did not
affect the prothrombin time (PT). However, although heparin dose-dependently increased the partial thromboplastin time (PTT) at therapeutic concentrations, SN7–13 had no activity, even at high concentrations (100 μg/ml).

**SN7–13 binds Tau with low nanomolar affinity**

We used a biolayer interferometry (BLI) kinetic binding assay (Octet, FortéBio) to analyze binding of Tau monomer to SN7–13 compared with heparin (Fig. 3 and Table 2). We biotinylated Tau monomer and immobilized it on super streptavidin biosensors, which were subsequently exposed to serial dilutions of heparin or SN7–13. Analysis of the binding kinetics indicated that heparin bound Tau with very high avidity, with an apparent \( K_D \) of 60 ± 30 nM (Fig. 3A). The interaction had a fast on rate (≈ \( 9 \times 10^5 \) M\(^{-1}\) s\(^{-1}\)) and a slow off rate (≈ \( 6 \times 10^{-5} \) M\(^{-1}\) s\(^{-1}\)). In comparison, SN7–13 bound to Tau with an apparent \( K_D \) of 2 ± 1 nM (Fig. 3B). The interaction had a fast on rate (≈ \( 1.6 \times 10^5 \) M\(^{-1}\) s\(^{-1}\)) and a slow off rate (≈ \( 3.1 \times 10^{-4} \) M\(^{-1}\) s\(^{-1}\)), qualitatively similar to heparin. In summary, SN7–13 demonstrated high binding efficiency for Tau, albeit slightly weaker than heparin.

**SN7–13 blocks Tau uptake in HEK293T cells**

We have previously observed that heparin prevents the binding of Tau to HSPGs on the cell surface, blocking aggregate uptake via HSPG-mediated macropinocytosis (3, 4). To test the efficacy of SN7–13, we labeled recombinant Tau fibrils with Alexa 647 dye and incubated them with HEK293T cells in culture for 4 h, titrating concentrations of heparin and SN7–13 (Fig. 4A). We quantified aggregate uptake by measuring the median fluorescence intensity (MFI) per cell using flow cytometry (3). Heparin reduced Tau uptake by ≈50% even at the lowest concentration tested (0.2 μg/ml). Higher concentrations of heparin decreased Tau uptake virtually to zero. SN7–13 had slightly lower potency but inhibited uptake similarly to heparin.
A growing number of studies suggest that Tau oligomers may play an important role in Tau pathology (13, 30). We therefore used established protocols to purify oligomeric assemblies (31). We sonicated labeled fibrils to create fragments and used SEC to purify oligomeric assemblies of defined sizes (3-mer, 10-mer, and 20-mer), with which we incubated with cultured HEK293T cells. Heparin and SN7–13 both inhibited oligomer uptake (Fig. 4B).

**SN7–13 blocks seeding with recombinant Tau**

Multiple pathways for cellular Tau uptake and downstream pathways have been proposed (1, 2), and some may lead to the generation of seeding in cells, whereas others may not. Therefore, we tested whether SN7–13 blocks Tau seeding in a well-characterized "biosensor" cell line. These cells stably express the Tau repeat domain (RD) containing the disease-associated mutation P301S, tagged with CFP or YFP (28, 29). When exposed to extracellular Tau fibrils, Tau aggregates bind to the cell surface, trigger their own uptake, and induce intracellular aggregation of Tau RD-CFP/YFP, bringing the fluorophores in close proximity and enabling FRET. We used FRET to measure intracellular seeding by flow cytometry. We exposed Tau fibrils to heparin or SN7–13 for 2 h prior to addition to the biosensor cells. These were incubated for 72 h and harvested for flow cytometry. Both heparin and SN7–13 dose-dependently reduced seeding, with similar potency (Fig. 5A). We next tested effects on oligomeric assemblies. We purified assemblies (3-mer, 10-mer, and 20-mer) as above and incubated them with biosensors along with heparin (200 µg/ml) or SN7–13 (20 and 200 µg/ml). Both compounds inhibited seeding by these species as efficiently as they inhibited seeding by fibrils (Fig. 5B).

**SN7–13 blocks Tau uptake in mouse hippocampal neurons**

HEK293T cells might have different HSPG content than neurons, and thus we tested whether SN7–13 would inhibit Tau uptake in primary cultured neurons. We isolated mouse hippocampal neurons from embryonic day 17.5 CD1 mice and cultured them in 96-well plates with 30,000 neurons/well. Between DIV 7 and 14, we exposed them to labeled Tau fibrils for 16 h and quantified Tau uptake by measuring the MFI via flow cytometry. Heparin and SN7–13 both dose-dependently reduced Tau uptake (Fig. 6), consistent with a common mode of HSPG binding in both cell types.

**SN7–13 blocks seeding by P301S transgenic mouse brain homogenate**

We have previously studied the progressive Tau seeding activity that develops in a transgenic mouse line (PS19) in which 1N4R human Tau containing the disease-associated mutation (P301S) is driven by the PrP promoter (29, 32, 33). We generated brain homogenate from ~ 400- to 450-day-old animals and applied it to the biosensor cells (33). We incubated heparin or SN7–13 with the brain homogenate for 2 h prior to addition to the cells. After 72 h we quantified intracellular aggregation by FRET flow cytometry (29). Heparin dose-dependently reduced seeding by brain homogenate. SN7–13 reduced seeding by brain homogenate similarly to heparin at higher concentrations (Fig. 7).

**Discussion**

We have previously proposed that transcellular propagation of Tau aggregates underlies progression of neurodegeneration in AD and related tauopathies (34). We have further determined that Tau binding to HSPGs at the cell surface mediates uptake and subsequent pathological conversion of native protein on the cell interior (3, 4). We have now developed a unique inhibitor of Tau uptake, SN7–13, that targets this mechanism. Although heparin and LMWH have been used in humans as potent anticoagulants for many decades (26, 27), the therapeutic window for both is relatively narrow, overdose can result in hemorrhage (8), and neither is a preferable agent for chronic use to inhibit transcellular propagation of Tau pathology.

Heparin is polydisperse with a molecular mass of 3–30 kDa (average 15 kDa). Its size and charge would be expected to prevent it from passing the blood–brain barrier (8). LMWH has a molecular mass of 2.5–8 kDa (average 4.5 kDa) (8), and preparations of heparin-derived oligosaccharides can pass the blood–brain barrier with CSF to plasma ratios of ~1% (35, 36).
Figure 4. SN7–13 inhibits uptake of Tau fibrils and oligomers in HEK293T cells. Recombinant Tau fibrils (A) labeled with Alexa Fluor 647 fluorescent dye were applied to HEK293T cells with increasing doses of heparin or SN7–13. Tau uptake was quantified based on the MFI per cell by flow cytometry. Heparin and SN7–13 dose-dependently decreased cellular uptake in both cell types. For uptake with Tau oligomers (B), Tau 3-, 10-, and 20-mer were purified using SEC, and labeled with Alexa Fluor 647 fluorescent dye. No inhibitor (labeled 0 μg/ml), heparin at 200 μg/ml (H200), or SN7–13 at 20 μg/ml (S20) and 200 μg/ml (S200) was simultaneously applied. Both heparin and SN7–13 efficiently inhibited uptake of Tau oligomers. For both A and B, each condition was recorded in triplicate. The values in A represent the average of three separate experiments. The values in B represent data from one experiment. The uptake in untreated samples was defined as 100%. The uptake in treated samples is shown relative to the untreated samples. The error bars indicate S.D.

Figure 5. SN7–13 blocks Tau seeding with Tau fibrils and oligomers. A, HEK293T biosensor cells were exposed to Tau fibril seeds in the presence of heparin or SN7–13. Heparin and SN7–13 efficiently inhibited seeding by recombinant Tau. B, Tau oligomers (3-, 10-, and 20-mer) were purified using SEC and applied to HEK293T biosensor cells. No inhibitor (labeled 0 μg/ml), heparin at 200 μg/ml (H200), or SN7–13 at 20 μg/ml (S20) and 200 μg/ml (S200) was simultaneously applied. Both heparin and SN7–13 efficiently inhibited seeding by Tau oligomers. For both A and B, each condition was recorded in triplicate. The values in A represent the average of three separate experiments. The values in B represent data from one experiment. The seeding in untreated samples was defined as 100%. The seeding in treated samples is shown relative to the untreated samples. The error bars indicate S.D.
were proposed as potential treatments (9, 14, 15, 39). Indeed, in cellular uptake and replication of PrP prions in cell culture and decades-old studies reported that sulfated glycans inhibit the binding of amyloid precursor protein (APP) to HSPGs (38). A LMWH, neuroparin, has exhibited neuroprotective activity in treated samples is shown relative to the untreated samples. The error bars indicate S.D. A recent study observed that heparin-like short oligosaccharides limit the cellular uptake of Tau oligomers and reduce toxicity in cell culture (13). Taken together, data from cell and animal studies have suggested that heparin derivatives could represent a valid therapeutic approach to block the cellular uptake of Tau and other prions and thus prevent the progression of pathology.

After our prior work determined a lower limit of GAG length that binds Tau (4), we decided to use a pentasaccharide analog of fondaparinux, a low-molecular-weight anticoagulant, as the starting molecule. We had previously established protocols to allow efficient and scalable synthesis of this compound (48, 49). Fondaparinux binds antithrombin (10, 11, 23). Three N-sulfate moieties play a critical role, because their deletion significantly reduces its anticoagulation properties (24, 25). We previously found that N-sulfation of GAGs mediates binding to Tau and is required for its cellular uptake (9). Surprisingly, we determined that replacing N-sulfate moieties with “linkable linkers” retained Tau binding and inhibitory activities similarly to heparin. We previously determined that the inhibitory potency of heparin derivatives on tau uptake increases with sugar chain length (25). Thus, we linked several pentasaccharides by carbon chains, extending the GAG length without increasing the overall charge of the molecule.

Ultimately, we used an analog of fondaparinux as a basic building block to synthesize an oligosaccharide composed of multiple pentasaccharide units connected by aliphatic linkers without N-sulfation. This ensured a sufficient GAG chain length for Tau binding and inhibition, and reduced the molecular mass and overall charge. We systematically created and tested seven generations of compounds. The final product was SN7–13, a polydisperse compound composed of 2–4 pentasaccharides with a molecular mass from 2.5 to 8 kDa. This molecular mass is significantly lower than the average molecular mass of standard heparin, which ranges from 3 to 30 kDa (26, 27).

Using biolayer interferometry, we found that SN7–13 binds to Tau monomer with high affinity. It should be noted that we used a 1:1 fitting model provided by the manufacturer (For-téBio) to determine the dissociation constant. This may not adequately reflect the binding kinetics because Tau most likely has more than one heparin-binding site (50, 51). In addition, affinity (dissociation and rebinding of single heparin or SN7–13 molecules to Tau) may affect our measurements and falsely prolong the dissociation, and this effect may be higher for heparin than for SN7–13 given the difference in molecular mass. Therefore, the relative difference of the true $K_D$ may be smaller between heparin and SN7–13 than reflected in the data. Although our studies cannot provide an absolute $K_D$ for Tau observed a possible extension of survival in patients with long-term intraventricular treatment with pentosan polysulfate (16, 43–45). Of note, shorter heparin-derived disaccharide units did not have anti-prion activity in cell assays (46), consistent with this report for Tau (4). We have previously determined that heparin inhibits cellular uptake in vitro and a polysulfated dextran derivative reduced neuronal Tau uptake in mice (3). We and others have found that the interaction of Tau aggregates with heparin derivatives depends on critical sulfate moieties on the sugar chain such as 6-O-sulfation (4, 47) and N-sulfation (4).

Figure 7. SN7–13 blocks Tau seeding with brain lysate. HEK293T biosensor cells were used to compare the activities of Heparin and SN7–13 as inhibitors of seeding with brain lysate from transgenic mice expressing P301S Tau (33). Heparin potently inhibited seeding with brain lysate. SN7–13 inhibited seeding at higher concentrations. Each condition was tested in triplicate. The values represent the averages of three separate experiments. The error bars indicate S.D.

The uptake in untreated samples was defined as 100%. The uptake in untreated samples is shown relative to the untreated samples. The error bars indicate S.D. Heparin and heparin derivatives have been previously studied for their potential to treat neurodegenerative disorders associated with prion protein, Tau, and Aβ. Early observations reported that LMWH and polysulfated compounds inhibit Aβ binding to heparin and HSPGs (36, 37) and that heparin inhibits the binding of amyloid precursor protein (APP) to HSPGs (38) in vitro. A LMWH, neuroparin, has exhibited neuroprotective effects in several animal models of AD (20), and similar effects were observed for enoxaparin (21, 22). For prion diseases, decades-old studies reported that sulfated glycanis inhibit the cellular uptake and replication of PrP prions in cell culture and were proposed as potential treatments (9, 14, 15, 39). Indeed, in rodents the peripheral application of polysulfated anions prevented the development of prion disease or significantly prolonged the incubation time (40–42). In addition, the intraventricular infusion of pentosan polysulfate, a sulfated GAG, significantly prolonged the incubation time and reduced neurodegenerative changes and infectivity in a mouse model of prion disease (17). Subsequently, in several case reports and two observational studies in small patient cohorts, researchers

LMWH has a longer half-life, better bioavailability at low doses, and a more predictable dose response than heparin (8).

Figure 6. SN7–13 inhibits Tau uptake in primary neurons. Recombinant Tau fibrils labeled with Alexa Fluor 647 were applied to mouse hippocampal neurons with increasing doses of heparin or SN7–13. Aggregate uptake was quantified by flow cytometry based on the MFI per cell. Heparin and SN7–13 dose-dependently decreased cellular uptake in both cell types. Each condition was recorded in triplicate, and the values represent the averages of three separate experiments. The values represent the averages of three separate experiments. The uptake in untreated samples was defined as 100%. The uptake in treated samples is shown relative to the untreated samples. The error bars indicate S.D. After our prior work determined a lower limit of GAG length that binds Tau (4), we decided to use a pentasaccharide analog of fondaparinux, a low-molecular-weight anticoagulant, as the starting molecule. We had previously established protocols to allow efficient and scalable synthesis of this compound (48, 49). Fondaparinux binds antithrombin (10, 11, 23). Three N-sulfate moieties play a critical role, because their deletion significantly reduces its anticoagulation properties (24, 25). We previously found that N-sulfation of GAGs mediates binding to Tau and is required for its cellular uptake (9). Surprisingly, we determined that replacing N-sulfate moieties with “linkable linkers” retained Tau binding and inhibitory activities similarly to heparin. We previously determined that the inhibitory potency of heparin derivatives on tau uptake increases with sugar chain length (25). Thus, we linked several pentasaccharides by carbon chains, extending the GAG length without increasing the overall charge of the molecule.

Ultimately, we used an analog of fondaparinux as a basic building block to synthesize an oligosaccharide composed of multiple pentasaccharide units connected by aliphatic linkers without N-sulfation. This ensured a sufficient GAG chain length for Tau binding and inhibition, and reduced the molecular mass and overall charge. We systematically created and tested seven generations of compounds. The final product was SN7–13, a polydisperse compound composed of 2–4 pentasaccharides with a molecular mass from 2.5 to 8 kDa. This molecular mass is significantly lower than the average molecular mass of standard heparin, which ranges from 3 to 30 kDa (26, 27).

Using biolayer interferometry, we found that SN7–13 binds to Tau monomer with high affinity. It should be noted that we used a 1:1 fitting model provided by the manufacturer (For-téBio) to determine the dissociation constant. This may not adequately reflect the binding kinetics because Tau most likely has more than one heparin-binding site (50, 51). In addition, affinity (dissociation and rebinding of single heparin or SN7–13 molecules to Tau) may affect our measurements and falsely prolong the dissociation, and this effect may be higher for heparin than for SN7–13 given the difference in molecular mass. Therefore, the relative difference of the true $K_D$ may be smaller between heparin and SN7–13 than reflected in the data. Although our studies cannot provide an absolute $K_D$ for Tau observed a possible extension of survival in patients with long-term intraventricular treatment with pentosan polysulfate (16, 43–45). Of note, shorter heparin-derived disaccharide units did not have anti-prion activity in cell assays (46), consistent with this report for Tau (4). We have previously determined that heparin inhibits cellular uptake in vitro and a polysulfated dextran derivative reduced neuronal Tau uptake in mice (3). We and others have found that the interaction of Tau aggregates with heparin derivatives depends on critical sulfate moieties on the sugar chain such as 6-O-sulfation (4, 47) and N-sulfation (4).

Figure 7. SN7–13 blocks Tau seeding with brain lysate. HEK293T biosensor cells were used to compare the activities of Heparin and SN7–13 as inhibitors of seeding with brain lysate from transgenic mice expressing P301S Tau (33). Heparin potently inhibited seeding with brain lysate. SN7–13 inhibited seeding at higher concentrations. Each condition was tested in triplicate. The values represent the averages of three separate experiments. The error bars indicate S.D.
binding to heparin and SN7–13, the results indicate that both compounds bind to Tau with high affinity. Cellular uptake and seeding assays are consistent with IC_{50} values in the low nanomolar range.

Importantly, we did not observe any toxicity in vivo by intraparenchymal injection of the compound in four independent studies in PS19 mice or chronic exposure to the compound via intraventricular pump delivery (supporting text and Figs. S1 and S2). None of the mice showed signs of hemorrhage at any time point of the studies. This is an important observation, because the routine anticoagulation tests (PTT and PT) may not always reflect anticoagulatory properties. Indeed, the anticoagulant fondaparinux (which was used to generate the basic building block of SN7–13) does not reliably affect the PTT/PT, and the U.S. Food and Drug Administration does not recommend either of these tests to monitor fondaparinux treatment (52).

After chronic administration, we did not observe effects on Tau pathology in vivo. Importantly, we could not perform detailed pharmacokinetic studies because of our failure to develop methods of MS detection of SN7–13. Thus it is possible that our pump infusion method produced very limited bioavailability. Additionally, we treated mice with pumps for 30 days, which may have been too short to achieve a measurable effect on Tau seeding. Given the limitations we faced in studying SN7–13 in vivo, additional studies are clearly required to test its efficacy more definitively.

In summary, we have created a novel molecule that exhibits several desirable characteristics as an inhibitor of Tau propagation. This work may assist future efforts to design heparin derivatives for the treatment of neurodegenerative disorders and other diseases such as cancer, infection, and inflammatory diseases that might respond to specific inhibition of protein binding to HSPGs (53).

Materials and methods

All experiments involving animals were approved by the University of Texas Southwestern Medical Center Institutional Animal Care and Use Committee.

Protein preparation, fibrillization, and labeling

Recombinant full-length WT Tau was purified and fibrillized as described previously (3). For uptake assays, fibrils were incubated with Alexa Fluor 647 succinimidyl ester dye (Invitrogen) for 1 h at room temperature (molar ratio monomer/dye 1:12.5), quenched with 100 mM glycine for 1 h at room temperature, and dialyzed overnight into PBS using dialysis cassettes (Thermo Scientific) to remove excess dye. The labeled fibrils were stored at 4 °C until use.

Synthesis and quality control of SN7–13

Synthesis of SN7–13 was performed at PharmaRen. 200 mg of a building block pentasaccharide and 400 mg of NaHCO₃ were dissolved in 8 ml of deionized H₂O. Separately, 252 mg of N,N-(disuccinimidyld) suberate was dissolved in 7 ml of dimethylformamide. 4 ml of this solution was added to the aqueous solution of the pentasaccharide derivative in four portions in 8 h. The mixture was allowed to stir at room temperature overnight. The next day, the remaining 3 ml of DMF solution was added in two portions, and the mixture was stirred for 4 h. The reaction mixture was evaporated and extracted with EtOAc (3 × 15 ml). The aqueous layer was evaporated to dryness and redissolved in 10 ml of deionized H₂O and purified on a Sephadex G 25 column (Sigma–Aldrich). The fractions that were stained blue by treatment with ammonium molybdate stain (TCI Chemicals) on a silica gel thin-layer plate were combined and evaporated to produce 140 mg of glassy off-white flakes (yield, 140 mg). The reproducibility of the described procedure was confirmed by repeating the synthesis three times at different scales following the protocol precisely. The compound was subsequently dissolved at 10 mg/ml in PBS and stored in aliquots at 4 °C until further use. SN7–13 is a polydisperse compound, and the molecule mass ranges from 2.5 to 8 kDa (Figs. 1 and 2). For the calculations of concentrations in our cell-based assays and the analysis of the kinetic studies in this paper, we assumed an average molecular mass of 5 kDa.

Coagulation studies

Coagulation studies were carried out at the clinical laboratory of Children’s Medical Center Dallas. To assess the anticoagulative properties of SN7–13, an in vitro spiking experiment was performed. Normal pooled plasma was used for spiking with increasing concentrations of SN7–13 and porcine unfractionated heparin (AMSBio). Normal pooled plasma was aliquoted into 1-ml microtubes. Increasing concentrations (0.01, 0.1, 1, 10, 20, 50, and 100 μg/ml) of SN7–13 and unfractionated heparin were added to the normal pooled plasma. The activated PTT and PT were analyzed within 30 min. The PTT and PT assays were performed on the STA-R Evolution (Diagnostica Stago, Parsippany-Troy Hills, NJ). The PT reagent used was STA Neoplastine CI Plus, and the PTT reagent was STA-PTT Automate reagent. An abnormal PTT was defined as a result outside the laboratory’s normal range (21.3–38.8 s). An abnormal PT was defined as outside the range of 12.0–15.3 s.

Biolayer interferometry

We used biolayer interferometry (Octet, FortéBio) to characterize the interaction of Tau monomer with SN7–13 versus heparin as a control. Assay buffer was composed of 10 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% BSA, and 0.01% Tween 20 in deionized water. Full-length WT Tau monomer was prepared as referenced above (3) and biotinylated with EZ-Link NHS-PEG4-Biotin (Thermo Scientific) according to the manufacturer’s instructions. For 500 µl of 1.656 mg/ml Tau monomer, the amount of 10 mM biotin solution was calculated using the following formula, where conc. is concentration, mol. mass is molecular mass, MCR is the molecular coupling ratio, and vol. is volume.

\[
\mu l \text{ of 10} \mu \text{mol biotin reagent} = \frac{1.656 \text{ mg/ml (conc.)}}{46 \text{ kDa (mol · mass)}} \times 5 \times 500 \mu l \text{ (vol · )}/10
\]

\[
= 9 \mu l \text{ (Eq. 1)}
\]

Tau monomer was incubated with the required amount of biotin for 60 min at room temperature. The reaction was
quenched using ZEBA spin columns 7000 molecular weight cutoff (Thermo Scientific) according to the manufacturer’s instructions and subsequently stored at −80 °C in small aliquots until use. For experiments, biotinylated Tau monomer was diluted with assay buffer to a concentration of 0.2 μM. In addition, biocytin (Sigma) was diluted in assay buffer to a stock solution of 1 μM. Super streptavidin biosensors (FortéBio) were equilibrated in 200 μl of assay buffer 20 min before use in a 96-well plate (Greiner). Meanwhile, 50 μl of assay buffer, biotinylated Tau fibrils, biocytin, and serial dilutions of heparin (10, 5, 2.5, 1.25, and 0.625 nm) and SN7–13 (200, 100, 50, 25, and 13 nm) were transferred to the designated wells on the 384-well assay plate, and the plate was centrifuged for 1 min at 1000 rpm. Binding assays were performed by loading the sensors with biotinylated Tau monomer, followed by quenching of streptavidin on the sensor with biocytin and equilibration in assay buffer, an association step (300 s), and a dissociation step (600 s) using an Octet RED 384 system (FortéBio, Pall Life Sciences) and the Octet 8.2 data acquisition software. The assays were performed at 30 °C with 1000 rpm shaking. Data from reference sensors (not loaded with Tau) was subtracted from sensors loaded with Tau to account for any nonspecific binding of heparin or SN7–13 to the sensors. In all experiments performed here, heparin and SN7–13 showed no nonspecific binding to unloaded Super Streptavidin sensors. The processed data were fit globally (across all dilutions within an experiment) to a 1:1 binding model to obtain kinetic and thermodynamic parameters. The data were processed and analyzed in the Octet 8.2 data analysis software, and the graphs were plotted in GraphPad Prism version 8 for Mac OS X.

Cell culture of HEK293T cells and P301S FRET biosensor cell line

We previously used HEK293T cells to generate a stable monoclonal P301S FRET biosensor cell line by overexpressing Tau RD with the disease-associated mutation P301S and tagged at the C terminus with either CFP or YFP to detect Tau seeding (ATCC CRL-3275) (28, 29). All HEK293T cells were grown in complete medium: Dulbecco’s modified Eagle’s medium (Gibco) with 10% fetal bovine serum (Sigma), 1% penicillin/streptomycin (Gibco), and 1% GlutaMAX (Gibco). The cells were cultured and passed at 37 °C, 5% CO2, in a humidified incubator. Dulbecco’s PBS (Life Technologies) was used for washing the cells prior to harvesting with 0.05% trypsin–EDTA (Life Technologies).

Tau uptake assay

Uptake assays were performed as described previously (4). The HEK293T cells were plated at 20,000 cells/well in a 96-well plate in 100 μl of medium/well. Fluorescently labeled aggregates (100 nm monomer equivalent) were sonicated for 30 s at an amplitude of 65 (corresponding to ~80 watts; Qsonica) and preincubated overnight at 4 °C in medium containing standard heparin (AMSBio) or SN7–13 at five different concentrations (0.2–500 μg/ml). The following morning, the aggregate–GAG complexes were applied to cells for 4 h in medium vehicles of 50 μl/well. The cells were harvested with 0.25% trypsin for 5 min, fixed with 2% paraformaldehyde for 10 min, and resuspended in flow cytometry buffer (HBSS plus 1% FBS and 1 mM EDTA) before flow cytometry. The cells were counted with the LSRFortessa SORP (BD Biosciences). We determined the MFI per cell to quantify cellular aggregate internalization. Each experiment was conducted in three independent biological replicates, with technical triplicates per condition. We determined the average MFI of the replicates for each condition and standardized to aggregate uptake without inhibitor treatment within each experiment. The standardized averages of each condition were then combined. Data analysis was performed using FlowJo v10 software (Treestar Inc.) and GraphPad Prism v8 for Mac OS X.

Tau seeding assay

We used the P301S FRET biosensor cell line for seeding experiments (28, 29). The seeding assay was conducted as described except that biosensor cells were plated at a density of 15,000 cells/well in a 96-well plate in a media volume of 100 μl/well. Recombinant Tau fibrils were sonicated for 30 s at an amplitude of 65 (corresponding to ~80 watts; Qsonica) prior to use. Fibrils (100 nm monomer equivalent) were preincubated overnight at 4 °C in medium containing the standard heparin (AMSBio) or SN7–13 at five different concentrations (0.2–500 μg/ml). At 20–30% confluency, the seed–heparin complexes were applied to the cells in medium volumes of 50 μl/well, and the cells were incubated for an additional 72 h. We did not use Lipofectamine to drive internalization of seeds because we sought to monitor HSPG-mediated uptake. The cells were harvested with 0.05% trypsin and fixed in 2% paraformaldehyde for 10 min and then resuspended in flow cytometry buffer (HBSS plus 1% FBS and 1 mM EDTA). The LSRFortessa SORP (BD Biosciences) was used to perform FRET flow cytometry. We quantified FRET as previously described (28, 29) with the following modification: we identified single cells that were YFP- and CFP-positive and subsequently quantified FRET-positive cells within this population. For each data set, three independent experiments with three technical replicates were performed. Data analysis was performed using FlowJo v10 software (Treestar Inc.) and GraphPad Prism v8 for Mac OS X.

Primary neuron culture

The day before preparation of the neurons, the plates were coated with 0.05% polyethyleneimine in 25 mM borate buffer, pH 8.4, at 37 °C overnight and washed three times with PBS the next day. Hippocampal neurons were isolated from embryonic day 17.5 CD1 mice as previously described (54). The neurons were incubated with 0.5% trypsin–EDTA (Gibco) in HBSS (Gibco) with 1:6 glucose (Sigma) for 20 min at 37 °C. The neurons were then triturated and filtered through a 70-μm filter (Greiner Bio-one; Easy strainer), followed by plating 30,000 cells/well of a 96-well plate in plating media: minimal essential medium (Gibco), 10% BSS (Gibco), 1 mM pyruvate (Gibco), 37% glucose (Gibco), and 1% penicillin–streptomycin (Gibco). After a 3-h incubation period, the medium was completely exchanged by 3-h exchange medium: neurobasal medium (Gibco), B27 (Gibco), 200 mM GlutaMAX (Gibco), and 25 mM glutamic acid (Sigma). After 3 days and from there on twice weekly, 50% of the media in each well was replaced with 3-day
were incubated with Tau fibrils for 16 h overnight, and the conducted similarly to the uptake assay in HEK293T cells Tau uptake assay in primary neurons.

Preparation of Tau oligomers for uptake and seeding

We used sonication and size-exclusion chromatography (SEC) to prepare Tau oligomers as described previously (50). For uptake assays, Tau fibrils were labeled with Alexa Fluor 647. For uptake assays, we used unlabeled fibrils. The fibrils were sonicated using a Q700 Sonicator (QSonica) at a power of 100–110 watts (amplitude 50) for a total of 2 min, and 1 ml of supernatant was loaded into a Superdex 200 Increase 10/300 GL column (GE Healthcare) and eluted in PBS.

Preparation of Tau oligomers for uptake and seeding

Uptake and seeding experiments were conducted as described above with the following difference: the aggregate–GAG complexes were applied in a media vehicle of 30 l/well (instead of 50 l/well; final Tau concentration, 10 nM).

References

1. Stopschinski, B. E., and Diamond, M. I. (2017) The prion model for progression and diversity of neurodegenerative diseases. Lancet Neurol. 16, 323–332 CrossRef Medline

2. Sanders, D. W., Kaufman, S. K., Holmes, B. B., and Diamond, M. I. (2016) Prions and protein assemblies that convey biological information in health and disease. Neuron 89, 433–448 CrossRef Medline

3. Holmes, B. B., DeVos, S. L., Kfoury, N., Li, M., Jacks, R., Yanamandra, K., Ouidja, M. O., Brodsky, F. M., Marasa, J., Bagchi, D. P., Kotzbauer, P. T., Miller, T. M., Papy-Garcia, D., and Diamond, M. I. (2013) Heparan sulfate proteoglycans mediate internalization and propagation of specific prionopathogenic seeds. Proc. Natl. Acad. Sci. USA 110, E3138–E3147 CrossRef Medline

4. Stopschinski, B. E., Holmes, B. B., Miller, G. M., Manon, V. A., Vaquero-Alicea, J., Prueitt, W. L., Hsieh-Wilson, L. C., and Diamond, M. I. (2018) Specific glycosaminoglycan chain length and sulfation patterns are required for cell uptake of Tau versus α-synuclein and β-amyloid aggregates. J. Biol. Chem. 293, 10826–10840 CrossRef Medline

5. Lindahl, U., and Kjellén, L. (2013) Pathophysiology of heparan sulfate: many diseases, few drugs. J. Intern. Med. 273, 555–571 CrossRef Medline

6. Xu, D., and Esco, J. D. (2014) Demystifying heparan sulfate–protein interactions. Annu. Rev. Biochem. 83, 129–157 CrossRef Medline

7. Alavi Naini, S. M., and Soussi-Yanicostas, N. (2018) Heparan sulfate as a therapeutic target in tauopathies: insights from zebrafish. Front. Cell Dev. Biol. 6, 163 CrossRef Medline

8. Hirsh, J., Warkentin, T. E., Shaughnessy, S. G., Anand, S. S., Halperin, J. L., Raschke, R., Granger, C., Ohman, E. M., and Dalen, J. E. (2001) Heparin and low-molecular-weight heparin: mechanisms of action, pharmacokinetics, dosing, monitoring, efficacy, and safety. Chest 119, 645–656 CrossRef Medline

9. Ben-Zaken, O., Tzaban, S., Tal, Y., Horonchik, L., Esko, J. D., Vlodavsky, I., and Taraboulos, A. (2003) Cellular heparan sulfate participates in the metabolism of prions. J. Biol. Chem. 278, 40041–40049 CrossRef Medline

10. Petitou, M., Casu, B., and Lindahl, U. (2003) 1976–1983, a critical period in the history of heparin: the discovery of the antithrombin binding site. Biochimie 85, 83–89 CrossRef Medline

11. Jin, L., Abrahams, J. P., Skinner, R., Petitou, M., Pike, R. N., and Carrell, R. W. (1997) The anticoagulant activation of antithrombin by heparin. Proc. Natl. Acad. Sci. USA 94, 14683–14688 CrossRef Medline

12. Erdal-Badju, E., Lu, A., Zuo, Y., Picard, V., and Bock, S. C. (1997) Identification of the antithrombin III heparin binding site. J. Biol. Chem. 272, 13993–14000 CrossRef Medline

13. Wang, P., Lo Cascio, F., Gao, J., Kayed, R., and Huang, X. (2018) Binding and neurotoxicity mitigation of toxic Tau oligomers by synthetic heparin like oligosaccharides. Chem. Commun. (Camb.) 54, 10120–10123 CrossRef Medline

14. Horonchik, L., Tzaban, S., Ben-Zaken, O., Yedidia, Y., Rovinsky, A., Papy-Garcia, D., Barrittault, D., Vlodavsky, I., and Taraboulos, A. (2005) Heparan sulfate is a cellular receptor for purified infectious prions. J. Biol. Chem. 280, 17062–17067 CrossRef Medline

15. Schönberger, O., Horonchik, L., Gabizon, R., Papy-Garcia, D., Barrittault, D., and Taraboulos, A. (2003) Novel heparan mimetics potently inhibit the scapie prion protein and its endocytosis. Biochem. Biophys. Res. Commun. 312, 473–479 CrossRef Medline

16. Tsuibo, Y., Doh-Ura, K., and Yamada, T. (2009) Continuous intraventricular infusion of pentosan polysulfate: clinical trial against prion diseases. Neuropathology 29, 632–636 CrossRef Medline

17. Doh-ura, K., Ishikawa, K., Murakami-Kubo, I., Sasaki, K., Mohri, S., Race, R., and Iwaki, T. (2004) Treatment of transmissible spongiform encephalopathy by intraventricular drug infusion in animal models. J. Virol. 78, 4999–5006 CrossRef Medline

18. Dudas, B., Cornelli, U., Lee, J. M., Hejna, M. J., Balzer, M., Lorenz, S. A., Mervis, R. F., Fareed, J., and Hanin, I. (2002) Oral and subcutaneous administration of the glycosaminoglycan C3 attenuates Aβ(25–35)-induced abnormal Tau protein immunoreactivity in rat brain. Neurobiol. Aging 23, 97–104 CrossRef Medline

19. Balzer, M., Lorenz, S., Hejna, M., Fareed, J., Hanin, I., Cornelli, U., and Lee, J. M. (2002) Low molecular weight glycosaminoglycan blockade of β-amyloid induced neuropathology. Eur. J. Pharmacol. 445, 211–220 CrossRef Medline

20. Dudas, B., Rose, M., Cornelli, U., Pavlovich, A., and Hanin, I. (2008) Neurrotrophic properties of glycosaminoglycans: potential treatment for
neurodegenerative disorders. *Neurodegener Dis.* 5, 200–205 CrossRefMedline
21. Bergamaschini, L., Rossi, E., Storini, C., Pizzimenti, S., Distaso, M., Perego, C., De Luigi, A., Vergani, C., and De Simoni, M. G. (2004) Peripheral treatment with enoxaparin, a low molecular weight heparin, reduces plaques and β-amloid accumulation in a mouse model of Alzheimer’s disease. *J. Neurosci.* 24, 4181–4186 CrossRefMedline
22. Timmer, N. M., van Dijk, L., van der Zee, C. E., Kiliaan, A., de Waal, R. M., and Verbeek, M. M. (2010) Enoxaparin treatment administered at both early and late stages of amyloid β deposition improves cognition of APPswg/PS1dE9 mice with differential effects on brain Aβ levels. *Neurobiol. Dis.* 40, 340–347 CrossRefMedline
23. Petitou, M., Lormeau, J. C., and Choay, J. (1991) A new synthetic pentasaccharide with increased anti-factor Xa activity: possible role for anionic clusters in the interaction of heparin and antithrombin III. *Semin. Thromb. Hemost.* 17, 143–146 Medline
24. Desai, U. R., Petitou, M., Björk, L., and Olson, S. T. (1998) Mechanism of heparin activation of antithrombin. Role of individual residues of the pentasaccharide activating sequence in the recognition of native and activated states of antithrombin. *J. Biol. Chem.* 273, 7478–7487 CrossRefMedline
25. Lapierre, F., Holme, K., Lam, L., Tessler, R. J., Storm, N., Wee, J., Stack, R. I., Castellot, J., and Tyrell, D. J. (1996) Chemical modifications of heparin that diminish its anticoagulant but preserve its heparanase-inhibitory, angiostatic, anti-tumor and anti-metastatic properties. *Glycobiology* 6, 355–366 CrossRefMedline
26. Hemker, H. C. (2016) A century of heparin: past, present and future. *J. Thromb. Haemost.* 14, 2329–2338 CrossRefMedline
27. Fareed, J., Hoppensteadt, D. A., and Bick, R. L. (2000) An update on heparins at the beginning of the new millennium. *Semin. Thromb. Hemost.* 26, 5–21 CrossRefMedline
28. Furman, J. L., Holmes, B. B., and Diamond, M. I. (2015) Sensitive detection of proteopathic seeding activity with FRET flow cytometry. *J. Vis. Exp.* e53205 CrossRefMedline
29. Holmes, B. B., Furman, J. L., Mahan, T. E., Yamasaki, T. R., Mirbaha, H., Eades, W. C., Belagyorod, L., Cairns, N. J., Holtzman, D. M., and Diamond, M. I. (2014) Proteopathic Tau seeding predicts tautauopathy in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 111, E4376–E4385 CrossRefMedline
30. Shafiei, S. S., Guerrero-Muñoz, M. I., and Castillo-Carranza, D. L. (2017) Tau oligomers: cytotoxicity, propagation, and mitochondrial damage. *Front. Aging Neurosci.* 9, 83 Medline
31. Mirbaha, H., Holmes, B. B., Sanders, D. W., Bieschke, J., and Diamond, M. I. (2015) Tau trimers are the minimal propagation unit spontaneously internalized to seed intracellular aggregation. *J. Biol. Chem.* 290, 14893–14903 CrossRefMedline
32. Kaufman, S. K., Thomas, T. L., Del Tredici, K., Braak, H., and Diamond, M. I. (2014) Experimental treatments for human transmissible spongiform encephalopathies. *Lancet* 383, 117 CrossRefMedline
33. Farquhar, C., Dickinson, A., and Bruce, M. (1999) Proplyphatic potential of pentosan polysulphate in transmissible spongiform encephalopathies. *Acta Neuropathol. Commun.* 41, 989–995 CrossRefMedline
34. Rauch, J. N., Chen, J. J., Sorum, A. W., Miller, G. M., Sharf, T., See, S. K., Hsieh-Wilson, L. C., Kampmann, M., and Kosik, K. S. (2018) Tau internalization is regulated by 6-O-sulfation on heparan sulfate proteoglycans (HSPGs). *Sci. Rep.* 8, 6382 CrossRefMedline
35. Payal Parth Patel, C. M., Kevin, K., Ohr, and Souren, N. (2013) Efficient and Scalable Process for the Manufacture of Fondaparinux Sodium, Reliable Biopharmaceutical Co., Overland, MO
36. Sourena Nadji, J. T. S., and Joseph Van A Artsdalen (2015) Synthetic heparinoid blocks Tau aggregate cell uptake. *J. Biol. Chem.* 284, 12845–12852 CrossRefMedline
37. Leveugle, B., Ding, W., Laurence, F., Dehouck, M. P., Scanameo, A., Cecchelli, R., and Fillit, H. (1998) Heparin oligosaccharides that pass the blood-brain barrier inhibit β-amyloid precursor protein secretion and heparin binding to β-amloid peptide. *J. Neurochem.* 70, 736–744 CrossRefMedline
38. Farber, J. L., Holm, B. B., and Diamond, M. I. (2015) Characterization of Tau prion seeding activity and strains internalized to seed intracellular aggregation. *J. Neurochem.* 1389–1392 CrossRefMedline
39. Caughey, B., and Raymond, G. J. (1993) Sulfated polyanion inhibition of scrapie-associated PrP accumulation in cultured cells. *J. Virol.* 67, 643–650 CrossRefMedline
40. Diringer, H., and Ehlers, B. (1991) Chemoprophylaxis of scrapie in mice. *J. Gen. Virol.* 72, 457–460 CrossRefMedline
41. Lagodana, A., Casaccia, P., Brogros, L., Cibati, M., Salvatore, M., Xi, Y. G., Masullo, C., and Pocchiari, M. (1992) Sulphate polyanions prolong the incubation period of scrapie-infected hamsters. *J. Gen. Virol.* 73, 661–665 CrossRefMedline
42. Farquhar, C., Dickinson, A., and Bruce, M. (1999) Proplyphatic potential of pentosan polysulphate in transmissible spongiform encephalopathies: is there a role for pentosan polysulphate? *Expert Opin. Biol. Ther.* 7, 713–726 CrossRefMedline
43. Parry, A., Baker, I., Stacey, R., and Wimalaratna, S. (2007) Long term survival in a patient with variant Creutzfeldt-Jakob disease treated with intravitreous pentosan polysulphate. *J. Neurol. Neurosurg. Psychiatry* 78, 733–734 Medline
44. Farb, J. N., Chen, J. J., Sorum, A. W., Miller, G. M., Sharf, T., See, S. K., Hsieh-Wilson, L. C., Kampmann, M., and Kosik, K. S. (2018) Tau internalization is regulated by 6-O-sulfation on heparan sulfate proteoglycans (HSPGs). *Eur. J. Org. Chem.* 2017, 4870–4877 CrossRefMedline
45. Payal Parth Patel, C. M., Kevin, K., Ohr, and Souren, N. (2013) Efficient and Scalable Process for the Manufacture of Fondaparinux Sodium, Reliable Biopharmaceutical Co., Overland, MO
46. Sourena Nadji, J. T. S., and Joseph Van A Artsdalen (2015) Synthetic heparinoid blocks Tau aggregate cell uptake. *J. Biol. Chem.* 284, 12845–12852 CrossRefMedline
47. Rauch, J. N., Chen, J. J., Sorum, A. W., Miller, G. M., Sharf, T., See, S. K., Hsieh-Wilson, L. C., Kampmann, M., and Kosik, K. S. (2018) Tau internalization is regulated by 6-O-sulfation on heparan sulfate proteoglycans (HSPGs). *Sci. Rep.* 8, 6382 CrossRefMedline
48. Payal Parth Patel, C. M., Kevin, K., Ohr, and Souren, N. (2013) Efficient and Scalable Process for the Manufacture of Fondaparinux Sodium, Reliable Biopharmaceutical Co., Overland, MO
49. Kauffman, S. K., Sanders, D. W., Thomas, T. L., Ruchinskas, A. J., Vaquer-Alicea, J., Sharma, A. M., Miller, T. M., and Diamond, M. I. (2016) Tau prion strains dictate patterns of cell pathology, progression rate, and regional vulnerability in vivo. *Neuron* 92, 796–812 CrossRefMedline
40. Kauffman, S. K., Sanders, D. W., Thomas, T. L., Ruchinskas, A. J., Vaquer-Alicea, J., Sharma, A. M., Miller, T. M., and Diamond, M. I. (2016) Tau prion strains dictate patterns of cell pathology, progression rate, and regional vulnerability in vivo. *Neuron* 92, 796–812 CrossRefMedline