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Binding and Neurotoxicity Mitigation of Toxic Tau Oligomers by Synthetic Heparin Like Oligosaccharides

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Abstract

Well-defined heparin like oligosaccharides up to decasaccharides were synthesized. It was discovered for the first time that heparin oligosaccharides, as short as a tetrasaccharide, can bind with the most toxic tau species, i.e., tau oligomers with nM K_D . The binding significantly reduced cellular uptake of the toxic tau oligomers and protected cells from tau oligomer induced cytotoxicity.

Graphical Abstract

Conflicts of interest
There are no conflicts to declare.

Heparin oligosaccharides can mitigate the cytotoxic effects of tau oligomers towards neuronal cells.

Alzheimer's disease (AD) is a progressive degenerative brain disease, which is estimated to affect 5.5 million Americans in 2017.¹ Although the causes for most AD cases have not been firmly established, the pathology of tau protein is believed to play a pivotal role in AD and other tauopathies.² While large molecular weight tau aggregates, known as neurofibrillary tangles (NFTs), are abundant in the brains of late stage AD patients,³ it is generally believed that these large insoluble aggregates may not be the key toxic species in AD.⁴ There are strong evidences supporting that the soluble, oligomeric tau (TauO) rather than the NFTs are likely the most toxic species causing disease and efficiently seeding the propagation of the pathology.^{5, 6} TauO have been found in the extracellular space around neurons in human AD patients.⁷ They can propagate through different brain regions, spreading neuronal death and associated learning and memory deficits.^{8, 9} Therefore, TauO are potential new therapeutic targets, and strategies that can reduce TauO-associated neurotoxicity are highly desired.

Heparan sulfate (HS) and its more sulfated analog heparin are a class of highly negatively charged polysaccharides present on mammalian cells including neuronal cells. HS and heparin are composed of repeating disaccharide subunits with D-glucosamine (GlcN) α -1,4 linked with a uronic acid (either L-uronic acid (IdoA) or D-glucuronic acid (GlcA)).^{10, 11} The amine moiety, 3-OH and 6-OH of GlcN and 2-OH of the uronic acid of heparin can be sulfated. With the positively charged proline-rich domain and the C-terminal tail in Tau protein,¹² heparin can bind with high molecular tau aggregates.¹³⁻¹⁵ However, it is not known whether heparin can interact with TauO. Herein, using structurally well-defined synthetic oligosaccharides, we report for the first time that heparin like oligosaccharides, as small as tetrasaccharides, can bind with the toxic TauO. Furthermore, treatment of cells with heparin like oligosaccharides protects them from TauO-induced toxicity providing an exciting new direction in targeting AD and related tauopathies.

In order to obtain heparin like oligosaccharides, we based our synthetic design on disaccharide modules **1**, **2** and **3**. Disaccharides **1** and **2** were synthesized starting from disaccharide **4**.¹⁶ For the non-reducing end disaccharide module, while TBS bearing disaccharide **4** could be used, we found it was impossible to remove the TBS group from sulfated oligosaccharides during deprotection.¹⁶ This consideration prompted us to prepare the 4'-O-benzyl (Bn) protected disaccharide **3**. Pre-activation of Bn protected glucosamine

donor **5** with *p*-TolSCl and AgOTf¹⁷ followed by the addition of acceptor **6**, gave the α -linked disaccharide **7** in 87% yield (Scheme 1a). The 1,2-*cis* linkage in the newly formed glycosidic bond was confirmed by NMR analysis ($J_{H1'-H2'} = 3.5$ Hz, $J_{H1'-C1'} = 171.0$ Hz). Protective group manipulation of **7** yielded the disaccharide module **3**. Reaction of the donor **3** with acceptor **1** generated tetrasaccharide **8** in 85% yield (Scheme 1b). Alternatively, **3** glycosylated the reducing end disaccharide module **2** giving tetrasaccharide **9** (Scheme 1b). In a similar manner, TBS bearing tetrasaccharide donor **10** was formed (Scheme 1c).

To produce the hexasaccharide backbone, a 4+2 glycosylation was carried out between the tetrasaccharide donor **8** and acceptor **2** leading to hexasaccharide **11** (Scheme 2a). The 4-*O*-TBS protected tetrasaccharide donor **10** also reacted well with disaccharide **2**. Removal of the TBS group from the glycosylation product produced the hexasaccharide acceptor **12** (Scheme 2b), which was subsequently glycosylated by tetrasaccharide donor **8**, forming decasaccharide **13** in 84% yield (Scheme 2c).

The deprotections and modifications of the backbones were carried out first by removal of 6-*O*-Lev from fully protected tetra-, hexa- and deca-saccharide **9**, **11** and **13** respectively with hydrazine acetate exposing the 6-OH (Scheme 3a). The conversion of these primary hydroxyl groups to carboxylic acids was mediated by bis(acetoxy)iodobenzene (BAIB) assisted 2, 2, 6, 6-tetramethyl-1-piperidinyloxy (TEMPO) oxidation.¹⁸ Since free carboxylic acids were found to lead to low yields in subsequent sulfation reactions,¹⁶ they were protected as either methyl (83% for tetrasaccharide **17** in 2 steps) or benzyl (77% for hexasaccharide **18** and 81% for decasaccharide **19** in 2 steps) esters. Removal of the acyl protecting groups was accomplished by treating oligosaccharides **17-19** with sodium methoxide, which gave **20**, **21** and **22** respectively (Scheme 3a).

The two azido groups in tetrasaccharide **20** were reduced by zinc powder to provide **23** with two free amine groups (Scheme 3b). Sulfations of free hydroxyls and amines of **23** were performed stepwise. Firstly, **23** was dissolved in methanol with aqueous NaOH solution adjusting the pH to 9.5 in order to deprotonate amine groups. *N*-sulfation was then performed by adding excess SO₃·Et₃N complex to the mixture to give **24** in 78% yield, which was then subjected to *O*-sulfation with SO₃-pyridine complex in pyridine overnight at 55 °C. Subsequent hydrogenolysis and saponification produced sulfated tetrasaccharide **25**.

For heparin hexasaccharide synthesis, the azido groups in **21** were first reduced to yield amine **26** (Scheme 3c). Similar stepwise sulfation as in synthesis of tetrasaccharide **25** was attempted on hexasaccharide **26**, which only led to decomposition of the starting materials. Analysis of the reaction mixture showed the formation of side products due to β -elimination with the oligosaccharide backbone cleaved. Instead, treatment of hexasaccharide **26** with 600 mM SO₃-py in pyridine at 55 °C successfully installed both *N*- and *O*-sulfation in one step. Interestingly, the free amine **26** obtained through 1, 3-propanedithiol reduction¹⁶ of **21** gave higher yields during sulfation compared to the corresponding compound produced by zinc power reduction. Subsequent catalytic hydrogenation and methyl ester hydrolysis gave the final heparin like hexasaccharide **27** at 64% yield over 3 steps (Scheme 3c). Analogously, the heparin like decasaccharide **28** was synthesized with an overall yield of 42% from **22** (Scheme 3d).

With the synthetic oligosaccharides in hand, their binding with TauO was analysed. **25**, **27**, and **28** were biotinylated, immobilized on streptavidin coated biolayer interferometry (BLI) sensors and incubated with various concentrations of TauO. The sensorgrams showed that tetrasaccharide **25** could bind to TauO with a K_D value of 2.79×10^{-7} M. Increasing the backbone length of the oligosaccharide to hexa- and deca-saccharides led to enhancements in TauO binding, with K_D values of 1.41×10^{-7} M and 3.49×10^{-8} M for oligosaccharides **27** and **28**, respectively. These results suggest that glycans, as short as tetrasaccharide, can already exhibit significant interactions with TauO and longer oligosaccharide backbones enhance the binding.

With the establishment of binding, we evaluated the abilities of heparin like oligosaccharides to modulate the aggregation state and toxicity of TauO. Oligomeric tau species were incubated with and without oligosaccharides at room temperature for 16 hours under oligomerization conditions.¹⁹ Atomic force microscopy (AFM) was performed to visualize and characterize the morphology and aggregation state, which reflects the distinct effects of the compounds on TauO. Classically, TauO exist as homogeneous spherical structures (Fig. 2a), while in the presence of heparin like oligosaccharides (Fig. 2b-d), TauO were converted into larger aggregates.

Next, we evaluated the ability of the heparin like oligosaccharides to prevent TauO-induced cytotoxicity using human neuroblastoma SH-SY5Y purchased from American Type Culture Collection. Cells were incubated with tau alone or in the presence of heparin like oligosaccharides **25**, **27** and **28**, respectively. The cytotoxicity to SH-SY5Y cells was determined in a lactate dehydrogenase (LDH)-based assay, with the amount of LDH released into the culture media from damaged cells indicative of cellular cytotoxicity and cytolysis. Consistent with the idea that TauO being the more toxic tau species, TauO treatment of SH-SY5Y cells led to significantly higher LDH release, while cells incubated with the higher molecular aggregate tau fibrils released much less LDH (Fig. 3a). Excitingly, in the presence of oligosaccharides (10 μ M), TauO induced significantly lower levels of LDH release, compared to TauO alone (Fig. 3a). Moreover, cells exposed to each condition were evaluated for morphological differences. In the presence of TauO alone, there was significant cell shrinkage compared to either the control cells (Ctrl) or cells treated with TauO in the presence of heparin like oligosaccharides (Fig. 3b). Taken together, these results suggest that heparin like oligosaccharides interact with and subsequently convert the toxic TauO into larger aggregates modulating their toxicity.

To further confirm our findings and gain a better understanding of the protective effects of the oligosaccharides, SH-SY5Y cells were incubated with sub-lethal concentration of TauO and imaged by confocal microscopy. TauO were observed in large areas of the cytoplasm of cells, indicating extensive cellular internalization of TauO (Fig. S1a). Intracellular TauO can instigate mitochondrial damage, induce cytochrome c release and stimulate reactive oxygen species production, which are potential mechanisms for their cytotoxicity.^{20, 21} SH-SY5Y cells treated with TauO, co-incubated with oligosaccharide **25**, **27** and **28**, showed a significant reduction in the percentage of area positive of TauO staining (Fig. S1b). These results indicate that the glycan-induced aggregates are less prone to be uptaken by the cells explaining their reduced cytotoxicity. Altogether, our findings suggest that the glycans

interact with TauO leading to the formation of larger tau aggregates and inhibiting them from cellular internalization.

In conclusion, with the increasing appreciation of the high toxicity of TauO, finding an effective prevention and treatment strategy available for tauopathies, has becoming important. In this work, using fully synthetic well-defined glycans, we report the new finding that heparin like oligosaccharide bearing 2-*O*, 6-*O* and *N*-sulfation can bind strongly with the most toxic tau species, i.e., TauO. Heparin oligosaccharides can convert TauO into less toxic high molecular weight species, and mitigate TauO-associated cytotoxicity. In addition, the glycans significantly reduce TauO cellular internalization, which is critical for the progression of the pathology. While heparin is known to bind with high molecular tau aggregates,^{13–15} this is the first time that heparin oligosaccharide is shown to interact with TauO. Importantly, the newly synthesized heparin oligosaccharides could aid in the development of novel therapeutic approaches for AD and related tauopathies. Further studies are ongoing to establish the impacts of heparin structures such as sulfation patterns and backbone sequences on TauO interactions.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Sensograms of heparin like oligosaccharide binding with TauO.

Fit curves of interactions between BLI sensors loaded with a) **25**, b) **27** and c) **28** and TauO at various concentrations were obtained using models from Octet Data Analysis 9.0.0.12. Tetrasaccharide **25** exhibited significant binding to TauO, with longer backbone length leading to stronger binding.

Fig. 2. Morphology characterization of TauO alone or in the presence of heparin like oligosaccharides.

Atomic Force Microscopy images of TauO without (a) and with oligosaccharides b) **25**, c) **27**, and d) **28** (5X). AFM images show the ability of the glycans to modulate TauO aggregation states converting TauO into much larger aggregates. Scale bars = 100 nm.

Fig. 3.

(A) SH-SY5Y cells cytotoxicity after exposure to 2 μ M TauO, or 2 μ M TauO with 10 μ M of oligosaccharide (**25**, **27**, **28**) and untreated control (**Ctrl**). Treatment of SH-SY5Y cells with TauO had significantly higher LDH release compared to the untreated control, cells exposed to TauO in the presence of oligosaccharides or cells exposed to high molecular weight tau fibrils. Each experiment was performed in triplicate ($n = 3$). Data were compared by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test: Ctrl vs TauO, **25**, **27**, and **28**, Fibrils: **** $p < 0.0001$, ** $p < 0.01$; TauO vs **25**, **27**, and **28**: §§§§ $p < 0.0001$. Bars and error bars represent means and standard deviations performed. (B) Untreated SH-SY5Y cells were compared to cells treated for 24 hours with TauO alone or TauO in the presence of oligosaccharides (**25**, **27**, and **28**) and evaluated for morphological changes. Scale bar = 20 μ m.

Scheme 1.
Synthesis of tetrasaccharide backbones **8–10**

Scheme 2.

Constructions of heparin hexa- and deca-saccharide backbones.

Scheme 3.

Deprotection and sulfation of heparin oligosaccharides.