Inhibition of Apolipoprotein E-Related Neurotoxicity by Glycosaminoglycans and Their Oligosaccharides†

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ABSTRACT: Apolipoprotein E (apoE) has been genetically linked to late-onset Alzheimer's disease (AD). The role of this lipid-transport protein in AD remains to be established. One hypothesis is that apoE, particularly the apoE4 isoform, may have neurotoxic effects as demonstrated using apoE-related synthetic peptides and the N-terminal fragment of apoE. ApoE is a heparan-sulfate binding protein, and apoE peptide neurotoxicity can be blocked by heparin and prevented by degrading heparan sulfate or inhibiting its biosynthesis. The possibility that heparin inhibition of toxicity is mediated by a specific oligosaccharide sequence was investigated using a bioassay to determine the inhibition of apoE peptide toxicity by glycosaminoglycans and purified glycosaminoglycan oligosaccharides. Studies on modified heparins showed that the presence of *N*-sulfo groups and either 2- or 6-*O* sulfo groups were required for inhibition of toxicity. Heparin oligosaccharides with eight or more saccharide residues with seven *O*-sulfo groups and four *N*-sulfo groups exhibited potent inhibition. Larger oligosaccharides, and heparin and heparan sulfate polymers, afforded comparable, or somewhat better, protective effects but also caused clumping and detachment of cells when administrated alone.

Alzheimer's disease (AD)[1] remains the most common form of dementia, with four million Americans currently suffering from it and an estimated 22 million people throughout the world who will be afflicted by 2025. There are no current effective treatments for AD, and the cause of this illness remains unknown despite the tremendous increase in information regarding genetic linkages to the disease. The pathological hallmarks of AD include the accumulation of extracellular plaques containing amyloid and other proteins, the presence of intracellular neurofibrillary tangles, whose effect on neuronal function is not yet known, and the loss of nerve cells and synaptic connections within the areas of the brain responsible for learning and memory. Mutations in three known genes, the amyloid protein precursor and presenilin 1 and 2, nearly always cause the disease (*1*). These mutations, however, account only for a very small percentage (5−10%) of AD cases. A fourth genetic factor is apolipoprotein E (apoE). Unlike the three other genes, the apoE4 gene is a susceptibility locus accounting for approximately 40−70% of the cases of late-onset AD (*2*).

ApoE is a 299-residue lipid-associated protein that binds and transports cholesterol-rich lipoproteins for internalization via receptors of the low-density lipoprotein (LDL) receptor family (*3*). In addition, apoE has other putative functions that do not seem to involve lipid transport (*4*). ApoE plays an important role in maintaining central nervous system functions (*4*, *5*), and recent studies have suggested that apoE is a major risk factor in a number of diseases (*6*). The N-terminal domain (1−191) is composed of four amphipathic α-helices arranged in an antiparallel fashion and connected by loop regions (*7*). It contains the low-density lipoprotein LDL receptor binding site (136−150) (*4*), and a high affinity heparin-binding site (142−147) overlapping with the receptor-binding region (*8*). The structure of the C-terminal domain (216−299) contains the major lipid-binding elements and is responsible for the self-association of apoE in the absence of lipid (*4*, *9*). It also contains a heparin-binding site only available for interaction in the lipid-free state (*8*).

The three major isoforms of the human apoE gene, apoE2, apoE3, and apoE4, are the products of three alleles at a single gene locus on chromosome 19, differing at amino acids 112 and 158 (*10*). ApoE3 (Cys-112, Arg-158) is the most common isoform. It binds to the LDL receptor and is associated with normal lipid metabolism. ApoE2 (Cys-112, Cys-158) displays defective binding and is associated with type III hyperlipoproteinemia (*11*). ApoE4 (Arg-112, Arg-158) is associated with a higher risk of heart disease (*12*,

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[1] Abbreviations: AD, Alzheimer's disease; apoE, apolipoprotein E; LDL, low-density lipoprotein; HSPG, heparan sulfate proteoglycan; LRP, low-density lipoprotein related receptor; ΔUAp, 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid; IdoAp, idopyranosyluronic acid; GlcAp, glucopyranosyluronic acid; GlcNp, 2-aminoglucopyranose; GalNp, 2-aminogalactopyranose; S, sulfate; MWCO, molecular weight cutoff; DP, degree of polymerization; SAX-HPLC, strong anion exchange high-performance liquid chromatography.

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13) and is the major genetic risk factor for sporadic AD (*13*−*16*).

Several hypotheses have been proposed to account for the isoform-specific association of apoE with AD (*17*). However, there is still no consensus regarding the role played by apoE in this, or other, neurodegenerative conditions. Most hypotheses assume that the apoE4 isoform is less effective than the apoE3 isoform in performing a positive function, such as (i) cytoskeletal stability through apoE binding to tau or other microtubule associated proteins (*18*), and tau phosphorylation (*19*), (ii) protective effects against neuronal injury through antioxidant activity (*20*), (iii) neuronal plasticity via effects on neurite outgrowth (*21*−*24*), and (iv) lipid peroxidation (*25*). ApoE has also been suggested to influence disease pathology via an indirect role through amyloid. For example, apoE has been postulated to modulate amyloid fibril formation, deposition, and/or clearance (*26*−*28*).

An alternative hypothesis is that apoE, especially the apoE4 isoform, contributes directly to neuropathology through neurotoxic effects. This possibility is supported by the findings that apoE-related synthetic peptides cause in vitro neurite degeneration and exhibit cytotoxicity (*29*), and the demonstration that full-length and truncated apoE exhibit neurotoxicity in an isoform-specific manner (apoE4 > apoE3) (*30*−*34*). A similar truncated apoE fragment was found in human brain and cerebrospinal fluid samples (*30*). All of the toxic apoE species include the receptor-binding region (*35*−*37*), as well as the overlapping high-affinity heparin-binding region (*9*). These neurotoxic effects are specific and appear to be mediated by a receptor complex that includes the low-density lipoprotein related receptor (LRP) and heparan sulfate proteoglycans (HSPG) (*38*, *39*): the neurotoxicity of apoE fragments is blocked when cultures of dissociated chick sympathetic neurons are pretreated with heparin lyase I, which degrades heparan sulfate, and sodium chlorate, which blocks glycosaminoglycan sulfation. In this HSPG-LRP pathway, apoE is thought to be localized to the cell surface through its interaction with HSPG and to be subsequently transferred to the LRP receptor for internalization.

Since HSPG is involved in the internalization of apoE through a binding step, compounds disrupting this binding, such as glycosaminoglycans or other highly charged polyanions, could be effective inhibitors of apoE neurotoxicity. In this study, we determined the ability of different glycosaminoglycans and their derivatives to inhibit apoE peptide neurotoxicity using a cell-based bioassay to investigate the relationship between structural properties and inhibitory activity.

MATERIALS AND METHODS

Depolymerization of Heparin. Porcine intestinal mucosa heparin (MW 18−23 kD, Celsus, Cincinnati, OH) was depolymerized with heparin lyase I from *Flavobacterium heparinium* (Sigma, St. Louis, MO) as previously described (*40*). Briefly, 276 mU of heparin lyase I was added to a solution of 3 g of heparin in 48 mL of 50 mM sodium phosphate buffer (pH 7.0) containing 0.2% BSA, and the reaction mixture was incubated at 30 °C. After 24 h incubation, another 276 mU of heparin lyase I was added to the reaction mixture. After an additional 24 h incubation, heparin lyase I was inactivated by adjusting the pH of the solution to 3.0 with concentrated HCl. The resulting reaction mixture was passed through a 10 × 0.5 cm sulfopropyl (SP-50)-Sephadex column (Sigma) to remove the proteins. The eluent was collected, and the pH was readjusted to 7.0. Residual heparin and high molecular weight oligosaccharides were removed by spin filtration through a 3000 MWCO membrane device (Millipore, Bedford, MA) spinning at 7000*g* for 30 min at 4 °C.

Gel Permeation Chromatography of the Low Molecular Weight Heparin Oligosaccharides. Small-scale chromatography was performed by eluting 2 mL of heparin digest on a 1.5 × 75 cm Biogel P6 (fine, Biorad, Hercules, CA) column with 0.5 M ammonium bicarbonate at a flow rate of 5 mL/h and collecting 1 mL fractions. Large-scale chromatography was performed by eluting 15 mL of heparin digest on a 2.5 × 120 cm Biogel P6 column at a flow rate of 20−25 mL/h and collecting 3.5-mL fractions. The absorbance of each fraction was measured at 232 nm and plotted versus the fraction number. Fractions corresponding to the same peak were pooled and repeatedly freeze-dried (three times) to remove ammonium bicarbonate. The resulting white powder was dissolved in 400 μL of distilled water, and the resulting suspension was filtered through a 0.22 μm filter (Millipore Product Division). The resulting eluent was further fractionated by SAX-HPLC.

Purification of Sized-Oligosaccharides by Semipreparative SAX-HPLC. Charge fractionation of each sized-oligosaccharide mixture was performed using a linear gradient of sodium chloride (0.2−2 M) adjusted to pH 3.5 with hydrochloric acid. The semipreparative column (5 μm Spherisorb, 2.5 × 25 cm, Waters, Millford, MA) was first equilibrated with 0.2 M NaCl and the sized-oligosaccharide fraction (50−100 mg) was applied to the column and eluted with a linear NaCl gradient at a flow rate of 4 mL/min over a period of 2 h. The elution profile was monitored by absorbance at 232 nm and the resulting peaks were manually collected and desalted by eluting up to 5 mL aliquots on seven Hi-Trap columns (Amersham Pharmacia Biotech, Piscataway, NJ) in series with distilled water at a flow rate of 1 mL/min. Absorbance of the resulting 1-mL fractions were measured at 232 nm, and the desalted oligosaccharide fractions were pooled together and freeze-dried. The resulting white powder was weighed and dissolved in 200 μL of distilled water, and the purity of the sample was checked by electrophoresis.

Analysis of Heparin Oligosaccharides by Electrophoresis. The purity of each oligosaccharide was assessed by polyacrylamide gel electrophoresis (32% polyacrylamide gel visualized with Azure A staining) (*40*) using a commercially available mixture of heparin oligosaccharides (Neoparin, San Leandro, CA), and by capillary electrophoresis using a P/ACE MDQ system (Beckman Coulter, Fullerton, CA). Capillary electrophoresis analyses were performed on a fused silica capillary (75 μm × 57 cm) under reverse polarity conditions with a constant current of 28 μA for 20 min at 20 °C using a 20 mM phosphoric acid buffer pH 3.5. Samples were injected by pressure injection (0.5 psi, 5 s). The percent purity was determined by peak area integration using the 32 Karat software (Beckman Coulter).

Structural Characterization of Pure Oligosaccharides. Pure oligosaccharides (∼1 mg) were dissolved in D_2O (99%) and freeze-dried to remove exchangeable protons. After three

exchanges, the sample was redissolved in 700 μL of D_2O (99.96%, Sigma). One-dimensional ^1H NMR experiments were performed on a Varian VXR-500 spectrometer equipped with a 5-mm triple-resonance tunable probe with standard software at 298K.

Cell Death Assay. To determine the extent of protection against apoE peptide neurotoxicity provided by heparin oligosaccharides, embryonic day 9 chick lumbar sympathetic ganglia were procured and cultured as previously described (*38*). Briefly, dissociated sympathetic neurons were plated onto poly-DL-ornithine coated 96-well plates. The cultures were incubated in a humidified environment with 5% CO_2 and 95% O_2 in Neurobasal medium (Invitrogen Life Technologies, Carlsbad, CA) overnight. On the following day, dissociated chick sympathetic cultures were transferred to F12 medium (Sigma) supplemented with ampicillin (Sigma). Dissociated chick cortical neurons were maintained in Neurobasal medium for all treatments and cultured for 20 h before being used for toxicity experiments. Potential inhibitors, porcine intestinal mucosa heparin (Celsus), bovine intestinal mucosa heparan sulfate M_w 18−23 kDa (Sigma), and porcine intestinal mucosa dermatan sulfate M_w 36 kDa (Celsus), chemically modified heparins (all from Neoparin, San Leandro, CA): oversulfated heparin, carboxyl reduced heparin, fully de-*O*-sulfated heparin, 2-*O*-desulfated heparin, 6-*O*-desulfated heparin, fully *N*-sulfated heparin, and de-*N*-sulfated heparin, enzymatically prepared heparin oligosaccharides of degree of polymerization (DP) 2, 4, 6, 8, 10, 12, 14, and 16, and dermatan sulfate oligosaccharides of DP2, 4, 6, and 8 (*42*) were added to the cell culture in the presence or absence of the apoE peptide (duplicated tandem sequence of apoE amino acids 141−149 prepared as previously described (*43*)) diluted in F12 medium. The proportion of living cells remaining after overnight incubation was determined by incubating the cultures with a vital dye (5-carboxyfluorescein diacetate, acetoxymethyl ester, Molecular Probes, Portland, OR) for 30−45 min at 37 °C. The vital dye was removed, and fresh unsupplemented F12 medium was added to the cultures. For quantification of cell number, the center of each well was visualized under fluorescent illumination (using a fluorescein filter) with the 4× objective of an Axiovert 20 inverted fluorescence microscope (Zeiss, Jena, Germany). A field covering ~15% (2.7 × 2.1 mm) of the total area of the well was captured with a video CCD camera linked to a Macintosh computer equipped with a Data Translation framegrabber card and running Image 1.62 software (National Institutes of Health). Images were captured from three to eight wells per treatment, the threshold set, and converted into a binary file, and the number of stained cells was counted by the computer using a macro developed for NIH Image. The number of surviving cells was averaged across the number of wells used for each treatment. This average was then used for comparison with the control wells from the same plate. Percent inhibition of toxicity was calculated using these internal plate controls, thus permitting comparisons across different plates. The addition of 8 μM of the peptide resulted in the death of 90% of the neurons. Significant protection ($p < 0.05$) could be reliably detected when cell numbers were within 30−100% of that obtained for untreated wells (ANOVA). Due to low variability across experiments, detailed statistical comparisons were not routinely required (*38*, *41*). If needed, ANOVA was carried out for treatment comparisons. With some treatments, clumping and/or detachment of cells occurred. This was especially true with larger oligosaccharides and with unmodified heparin. In such cases, the fluorescence of each well was also measured using a Cytofluor series 4000 fluorescence multiwell plate reader (Framingham, MA). This permitted quantification of cell viability that included any cells that might be floating in the medium or clumped together such that the cell counting macro would not resolve them as individual cells.

Activated Partial Thromboplastin Time (APTT). APTT was determined according to the manufacturer's specifications (Sigma) using heparin control, coagulation control, and oligosaccharide samples. A total of 0.1 mL of each of the controls or oligosaccharide samples was incubated at 37 °C for 1 min in a test tube. A total of 0.1 mL of warm APTT reagent was added to the tube and incubated for exactly 3 min, and 0.1 mL of warm 50 mM calcium chloride was rapidly expelled in the solution. Visual clot formation was determined and recorded.

RESULTS AND DISCUSSION

Previous work showed that heparin was able to protect neurons against apoE-related cytotoxicity at low concentrations (*38*, *43*). The mechanism of this inhibition is probably due to interference with the binding of apoE heparin-binding domains with cell surface HSPG. Compounds able to disrupt the binding between apoE and HSPG, such as glycosaminoglycans or other highly charged polyanions, are potentially effective inhibitors of apoE-related neurotoxicity.

Glycosaminoglycans display a number of groups that may contribute to protein binding. Besides carboxyl groups and hydroxyl groups, *N*- and *O*-sulfo groups are of particular importance. *N*-sulfo groups are located at the C-2 position of the GlcNp residues in heparin (86% *N*-sulfo, 14% *N*-acetyl) and heparan sulfate (10% *N*-sulfo, 90% *N*-acetyl), and *O*-sulfo groups at the C-6 position of GlcNp residues and at the C-2 position of IdoAp units. In addition, there is limited 3-*O*-sulfo group substitution on GlcNp residues of both heparin and heparan sulfate. In dermatan sulfate, there are no *N*-sulfo groups and the *O*-sulfo groups are found at the C-4 position of GalNp moieties. Compared to heparan sulfate, heparin is more highly sulfated and might contain more sulfo groups than required for the interaction, thus masking the essential binding sequence. Moreover, the actual binding sequence is usually contained within domains that are considerably shorter than full-sized heparin or heparan sulfate. To determine the negatively charged groups and the minimal size of the heparin domain involved in the interaction with apoE, two different strategies were used. First, native heparin, heparan sulfate, dermatan sulfate, and chemically modified heparins were tested as neurotoxicity inhibitors. Second, heparin and dermatan sulfate were depolymerized to afford a library of oligosaccharides with different lengths, sulfation, and uronic acid patterns, which were subsequently tested as potential inhibitors.

Potential neurotoxicity inhibitors were evaluated with a cell death bioassay using neurons obtained from embryonic chick sympathetic ganglia. The use of these primary neurons for screening inhibitors of apoE peptide toxicity has previously been shown to be reproducible and to parallel the

Table 1: Neurotoxicity Inhibition and APPT Values of Glycosaminoglycans and Chemically Modified Heparin

compound	IC_{50} (μM)	APPT (min)
heparin	0.2	>30
heparan sulfate	0.3	n.d.[a]
dermatan sulfate	1.3	n.d.
oversulfonated heparin	0.3	>30
fully *N*-sulfonated heparin	0.7	6
de-*O*-sulfonated heparin	1.6	1
2-*O*-desulfonated heparin	0.4	9
6-*O*-desulfonated heparin	0.5	7
N-desulfonated heparin	4.0	1
carboxy-reduced heparin	0.6	4

[a] n.d., not determined.

results obtained with mammalian neurons (*38*, *41*). The major advantage in using these neuronal cells is that they can be obtained in reasonably high numbers with greater than 95% homogeneity. This substantially reduces the variability obtained with mammalian neurons while also avoiding the potential difficulties associated with the use of transformed cells. This assay has previously been optimized to determine the extent of neuronal cell death following exposure to the apoE peptide over a period of 20 h, which provides an accurate estimate of the maximal extent of cell death (*38*). The use of a vital dye to visualize surviving cells has been found to be the most sensitive and reliable means of documenting cell death in this assay. In addition, use of a vital dye permits cytofluorometry, thereby providing accurate assessment of viability under conditions in which clumping or floating of cells occurs. The use of embryonic chick sympathetic ganglia also ensures a relatively homogeneous population of target neurons. The apoE peptide used in this study comprises two copies of amino acids 141−149 in tandem. Previous work has shown that the toxicity exhibited by this peptide shows similar properties to that exhibited by full-length and truncated apoE (*29*−*31*, *34*, *38*, *41*). Assessment of the extent of the toxicity and degree of protection of candidate inhibitors were carried out by automatically counting the surviving neurons with a fluorescence microscope and a fluorescence microplate reader. An example of results obtained with this assay is shown in Figure 4. The majority of neurons plated under these conditions survive during the 24 h treatment period, as shown in Figure 4A. Exposure to the apoE peptide results in the death of the majority of neurons (Figure 4B). The addition of a heparin octasaccharide (O2) did not cause toxicity and did not interfere with attachment of the neurons to the dish (Figure 4C). The same compound also completely prevented the neurotoxicity caused by the apoE peptide (Figure 4D). These results are representative of those obtained with other protective compounds.

Glycosaminoglycans and Chemically Modified Heparins. Initial screenings using heparin and heparan sulfate showed very good protection against apoE peptide toxicity with an IC_{50} of 0.2 and 0.3 μM, respectively (Table 1). Dermatan sulfate, although not as efficient as heparin, inhibited the neurotoxicity with an IC_{50} of 1.3 μM.

To assess the influence of the negatively charged groups (carboxyl and sulfo) toward the inhibition of apoE toxicity, seven chemically modified heparins were tested (Table 1). By employing oversulfonated heparin, carboxyl-reduced heparin, and selectively desulfonated heparins, we found that the groups having the largest influence on the inhibition are the *N*-sulfo groups, as demonstrated by the 20-fold reduction in potency following removal of these groups. However, replacing the *N*-acetyl groups (14% in heparin) by *N*-sulfo groups reduced the IC_{50} of heparin by 3.5-fold. This higher IC_{50} could be due to a conformational change of the heparin molecule induced by the excess of *N*-sulfo groups, thus resulting in a lower affinity binding between the *N*-sulfonated heparin and the apoE peptide. The importance of the *N*-sulfo groups for the inhibition of apoE peptide toxicity is in accordance with a recent study showing that *N*-sulfo groups are required for the formation of a high affinity complex between heparin and apoE4 (*44*). Selective 2-*O*-desulfonation, 6-*O*-desulfonation, and total *O*-desulfonation decreased the protective effect by 2-, 2.5-, and 8-fold, respectively. Although the influence of the 2-*O*-sulfo and 6-*O*-sulfo groups taken separately is not very pronounced, there appears to be synergism between these groups, possibly through a conformational effect. Reduction of the carboxyl groups to the corresponding hydroxymethyl groups reduced the inhibitory effect of heparin by 3-fold. This decrease in activity could indicate a direct binding between the carboxyl groups and the peptide, and/or could be the result of a conformational change in heparin. By reducing the carboxyl groups, the flexibility of the heparin molecule, which is in large part the result of the different conformations ($^1C_4$, $^2S_0$, and $^4C_1$) that iduronic acid residues can adopt (*45*), is also diminished. This lower flexibility could result in the weaker binding observed with carboxyl-reduced heparin. The importance of the conformation of the iduronic acid residues in relation to biological activity was recently demonstrated in a study with the antithrombin binding pentasaccharide, clearly explaining how the unique conformational behavior of iduronic acid translates to biological behavior (*46*). Oversulfonation of heparin did not improve the inhibition, indicating that simply providing an excess of negative charged groups does not lead to a tighter binding between the heparin molecule and the apoE peptide. The inhibition induced by dermatan sulfate (Table 1) was 6-fold lower than that displayed by heparin. Compared to heparin, dermatan sulfate has a reduced number of sulfo groups, with an average of one per disaccharide unit compared to 2.7 in heparin. The major uronic acid residue in dermatan sulfate, as in heparin, is IdoAp, but these residues do not contain a 2-*O*-sulfo group. Thus, the only *O*-sulfo groups in dermatan sulfate are located at the C-4 position of the GalNp residues. The hexosamine residue in dermatan sulfate is *N*-acetylated and, therefore, lacks an *N*-sulfo group. The reduced number of sulfo groups in dermatan sulfate, combined with the lack of the essential *N*-sulfo groups, might explain the lower activity of dermatan sulfate when compared with heparin.

All native and chemically modified glycosaminoglycans, with the exception of the *O*-desulfonated heparin, induced the formation of a large number of floating and clumping cells in the absence of the apoE peptide. This presumably reflects interference with cell attachment, which is largely mediated by cell surface charge. To avoid inaccurate conclusions regarding this effect, treatments that resulted in either floating or clumping of cells were evaluated using cytofluorometric analysis, which is not affected by such perturbations.

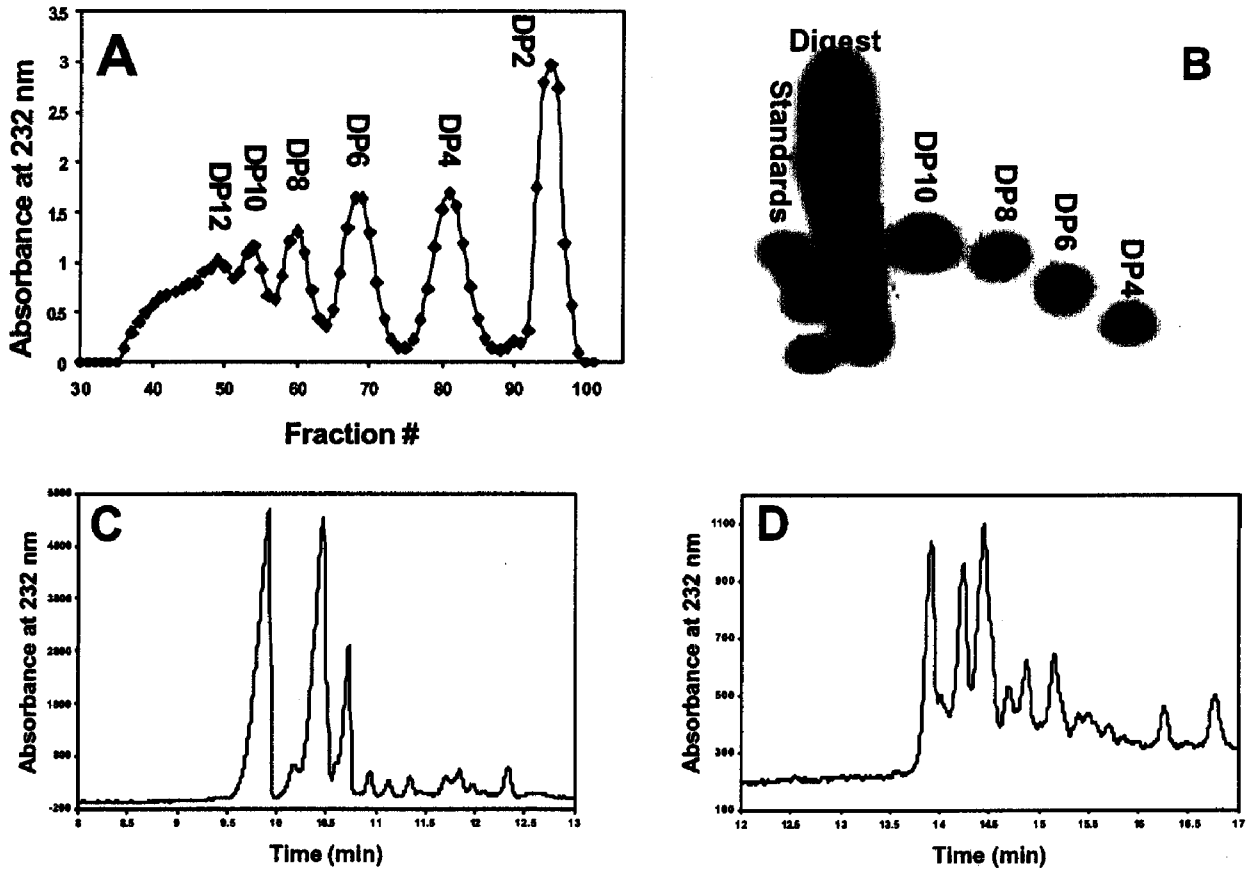

FIGURE 1: Preparation of size homogeneous heparin oligosaccharides: (A) Gel permeation of heparin digest; (B) gel electrophoresis analysis of DP fractions. The standard mixture contains commercial heparin-derived tetra-, hexa-, octa-, and decasaccharides; (C) capillary electrophoresis analysis of DP4 fraction; (D) capillary electrophoresis analysis of DP8 fraction.

Heparin and Dermatan Sulfate Oligosaccharides. Although heparin, heparan sulfate, and dermatan sulfate were found to be effective inhibitors of apoE peptide neurotoxicity, these glycosaminoglycans can also bind to a large number of other proteins and therefore, have a variety of other biological activities, e.g., anticoagulation. If the inhibition of toxicity by glycosaminoglycan is mediated by a specific oligosaccharide sequence, it would be expected that specific purified oligosaccharides derived from heparin would exhibit relatively specific inhibition of apoE peptide toxicity and weaker anticoagulant activity.

Heparin and dermatan oligosaccharides were enzymatically prepared using heparin lyase I and chondroitin B lyase, respectively. The controlled enzymatic depolymerization of heparin, followed by removal of proteins using SP−Sephadex chromatography and high molecular weight (M_w > 5000 kDa) oligosaccharides by pressure filtration, yielded a mixture of oligosaccharides ranging from disaccharides (DP2) to dodecasaccharides (DP12). Gel permeation chromatography of this low molecular weight oligosaccharide mixture (DP2−12) on Biogel P6 afforded size-homogeneous fractions of disaccharides up to dodecasaccharides (Figure 1A). The resulting DP fractions were repeatedly freeze-dried. The size homogeneity of each DP fraction was confirmed by gel electrophoresis (Figure 1B), and by a standard mixture of commercial heparin oligosaccharides (ranging from DP4 to DP10). The heterogeneity of each oligosaccharide fraction was characterized by capillary electrophoresis (Figure 1C,D). Capillary electrophoresis analysis is much faster than corresponding HPLC analysis (20 min versus 2 h) and allows a good estimation of the number of oligosaccharides present within each DP fraction. These sized-fractions were further fractionated by semipreparative SAX-HPLC using a NaCl gradient, and each peak was manually collected (Figure 2A−D). The resulting fractions were desalted on Hi-Trap columns, and their purity was checked by gel and capillary electrophoresis. Oligosaccharides having a purity greater than 90% were tested for inhibition of neurotoxicity. These separations resulted in the isolation of two pure tetrasaccharides (T1 and T2, Figure 3), two pure hexasaccharides (H1 and H2, Figure 3) and 2 pure octasaccharides (O1 and O2, Figure 3). For DP 10 and higher, only a "fully" sulfated oligosaccharide corresponding to the major sequence found in heparin was isolated and tested. The structure of each oligosaccharide was assigned by proton NMR and by comparison with known structures (*40*, *47*, *48*). These oligosaccharides were chosen because (i) they were available in >90% purity after the first HPLC fractionation; (ii) they represented the major components of each DP fraction; (iii) their structures were known and described in the literature; and (iv) they showed the highest activity. Other oligosaccharides with a purity ranging from 70 to 90% were also tested but were not as active.

Heparin disaccharide D2 (Figure 3) did not display any protective effect against the apoE peptide (data not shown). While the heparin tetrasaccharide T1 was also inactive, tetrasaccharide T2 having one additional *O*-sulfo group, had an IC_{50} of 49 μM (Figure 5). Hexasaccharide H1 showed an IC_{50} of 15 μM, while the more highly sulfated hexasaccharide H2 (9 sulfo groups in H2 versus 8 in H1) required only 8

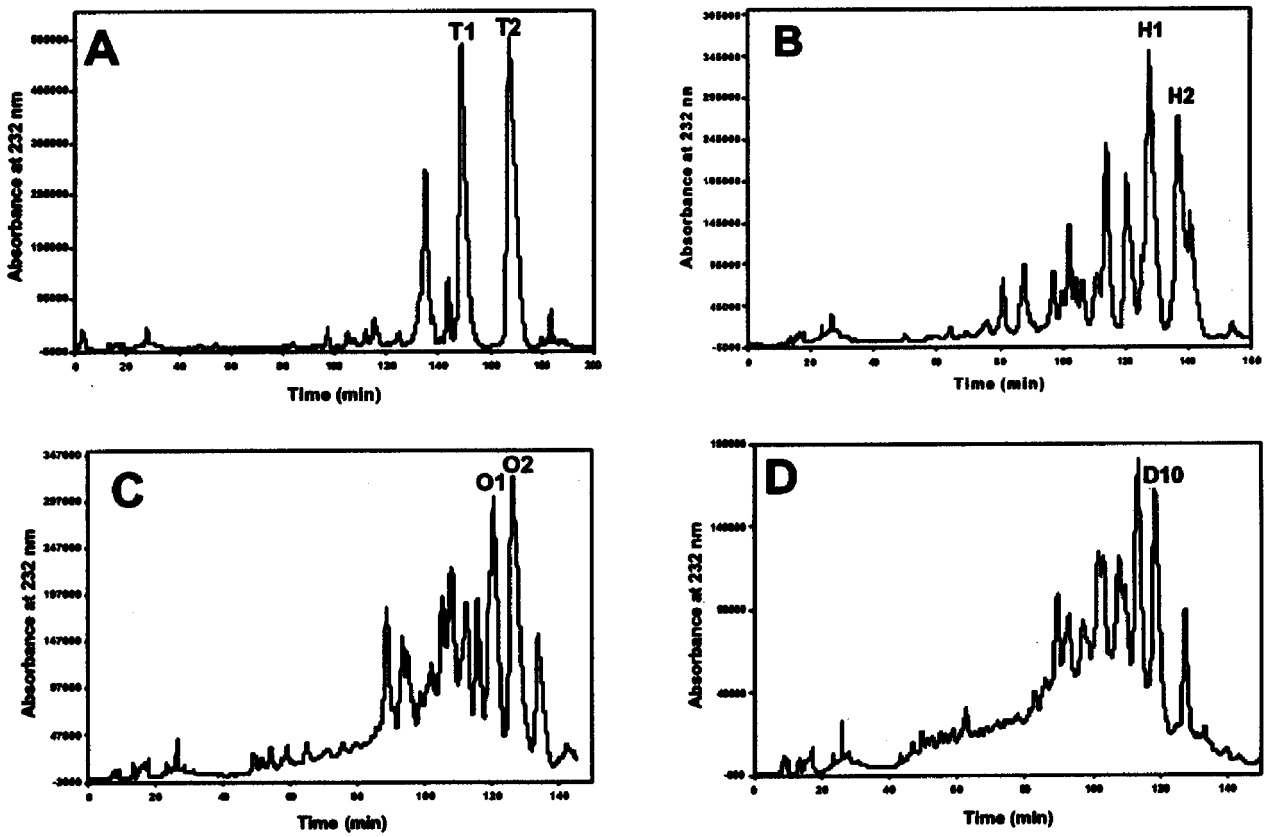

FIGURE 2: SAX-HPLC fractionation of sized heparin oligosaccharides: (A) DP4 fraction; (B) DP6 fraction; (C) DP8 fraction; (D) DP10 fraction.

μM to obtain the same half-inhibition. The octasaccharides O1 and O2 were four times more effective than hexasaccharide H2. Despite the difference in *O*-sulfo group content in these two octasaccharides (O1 with 7 and O2 with 8), both showed comparable IC_{50} values, 2.2 and 1.8 μM, suggesting that simply increasing *O*-sulfo group content does not improve inhibitory activity. For tetra-, hexa-, and octasaccharides, the oligosaccharides in which one of the IdoAp2S residue (in T2, H2, and O2) is substituted by a GlcAp residue (in T1, H1, and O1), displayed less inhibition, indicating that the 2-*O* sulfo group of iduronic acid may be involved in the binding of the apoE peptide. This observation is in agreement with the model proposed for the octasaccharide−apoE complex (*49*). The methyl glycoside of heparin pentasaccharide P5 (Figure 3) (*50*), corresponding to the active sequence of heparin binding antithrombin III, was also tested and showed a half-inhibition at 32 μM, an intermediate value between the IC_{50} of the tetrasaccharide T2 (49 μM) and the hexasaccharide H2 (8 μM). No floating and clumping cells were observed when purified tetrasaccharides through octasaccharides were tested. Testing of decasaccharide D10, dodecasaccharide D12, tetradecasaccharide D14, and hexadecasaccharide D16, resulted in a minimum IC_{50} value of 1.1 μM observed for D12 (Figure 5). In contrast to the smaller oligosaccharides examined, however, oligosaccharides D10, D12, D14, and D16 (*40*) all induced a large number of floating and clumping cells when tested alone. This effect was less pronounced when the concentration was dropped to the IC_{50} range.

Dermatan sulfate disaccharide (DS2, Figure 3), tetrasaccharide (DS4, Figure 3), hexasaccharide (DS6, Figure 3), octasaccharide (DS8, Figure 3), and decasaccharide (DS10, Figure 3) were also tested against the apoE peptide. Surprisingly, none of these oligosaccharides showed measurable activity. Extrapolation of the 15% cell survival numbers obtained at an oligosaccharide concentration of 250 μM indicated an IC_{50} in the range of several hundred micromolar, approximately 500-fold higher than that observed for dermatan sulfate. Modeling experiments of the heparin octasaccharide O2−apoE4 complex (*49*) suggest that the basic residues of the HSPG-binding site of apoE4 complemented all but one of the sulfo groups of this octasaccharide. Each 6-*O*-sulfo group of the GlcNpS6S residues, and each of the 2-*O*-sulfo groups of the IdoAp2S moieties were shown to interact with either an arginine or lysine from the HSPG-binding site. Similarly, there was an interaction with the 2-*N*-sulfo group of one of the GlcNpS6S units. This model suggests that lacking both the 2-*O*- and *N*-sulfo groups, and having *O*-sulfo group at the C-4 position of the hexosamine residues instead of the C-6 position, dermatan sulfate oligosaccharides are not able to interact as tightly with apoE as do heparin oligosaccharides.

Activated Partial Thromboplastin Time (APTT). The coagulation time determined for heparin and chemically modified heparins (Table 1) showed that chemical modification of heparin (sulfo group removal) greatly reduced anticoagulant activity. Furthermore, none of the heparin oligosaccharides examined, with the exception of the pentasaccharide, had an antithrombin pentasaccharide binding sequence and none displayed anticoagulant activity (*52*).

In summary, these results show that a heparin octasaccharide is the shortest oligomer capable of inhibiting apoE peptide toxicity in the low micromolar range. This observation is consistent with recent data showing that octasaccharide

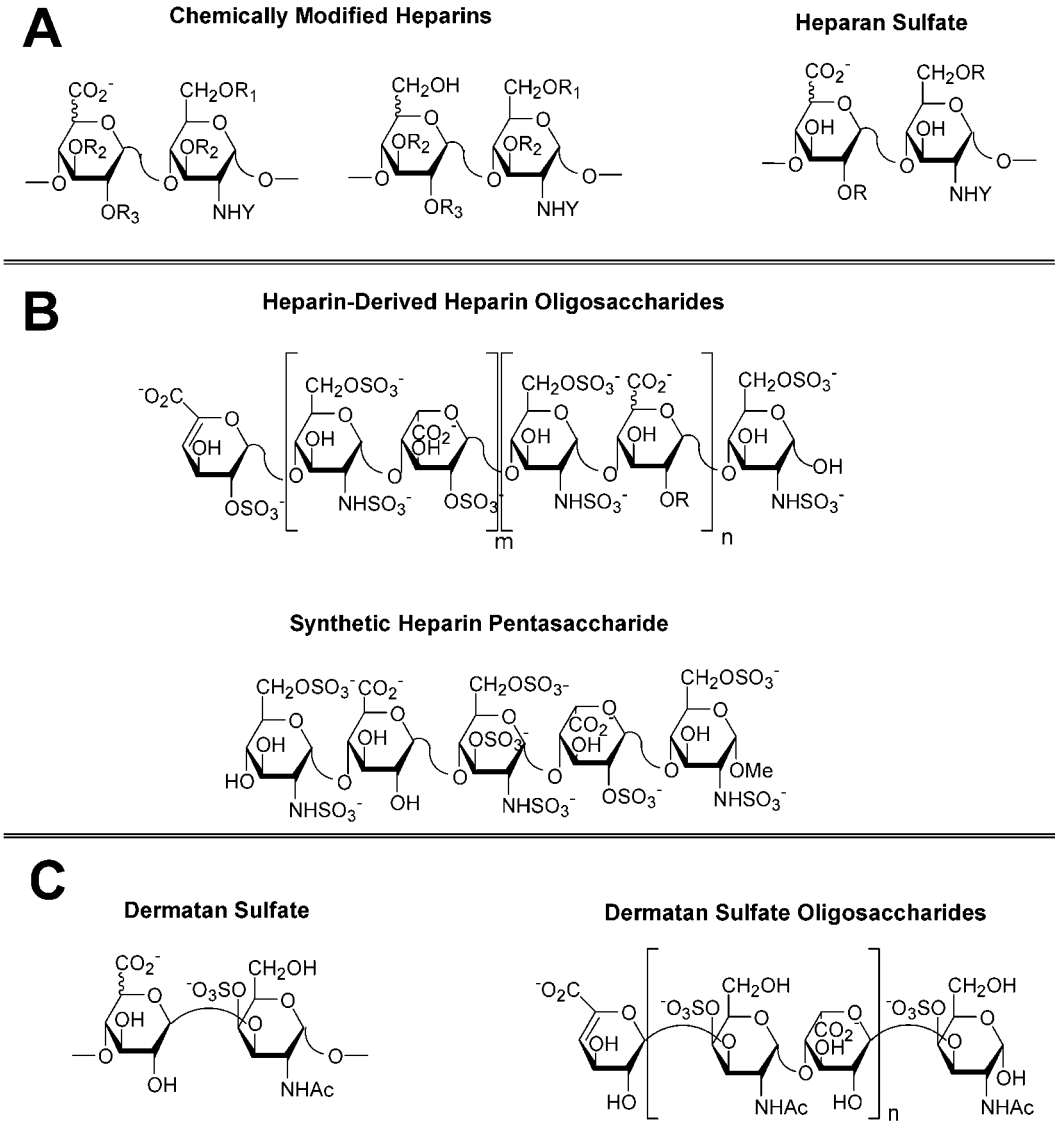

FIGURE 3: Structures of the candidate inhibitors. (A) Chemically modified heparins where the uronic acid residue is 90% iduronic acid and 10% glucuronic acid. Heparin $R_1 = R_3 = SO_3^-$, $R_2 = H$, $Y = 86\%$ SO_3^-/14% Ac; oversulfated heparin $R_1 = R_2 = R_3 = SO_3^-$, $Y = 86\%$ SO_3^-/14% Ac; de-*O*-sulfonated heparin $R_1 = R_2 = R_3 = H$, $Y = 86\%$ SO_3^-/14% Ac; 2-*O*-desulfonated heparin $R_1 = SO_3^-$, $R_2 = R_3 = H$, $Y = 86\%$ SO_3^-/14% Ac; 6-*O*-desulfonated heparin $R_1 = R_2 = H$, $R_3 = SO_3^-$, $Y = 86\%$ SO_3^-/14% Ac; *N*-sulfonated heparin $R_1 = R_3 = Y = SO_3^-$, $R_2 = H$; de-*N*-sulfonated heparin $R_1 = R_3 = SO_3^-$, $R_2 = H$, $Y = 86\%$ H/14% Ac; and carboxyreduced heparin, $R_1 = R_3 = SO_3^-$, $R_2 = H$, $Y = 86\%$ SO_3^-/14% Ac where the iduronic acid has been reduced to idose. Heparan sulfate where the uronic acid residue is 23% iduronic acid and 77% glucuronic acid, $R = 90\%$ H/10% SO_3^-, $Y = 16\%$ SO_3^-/84% Ac. (B) Heparin-derived heparin oligosaccharides. Where the uronic acid residue closest to the reducing end is glucuronic acid and R = H: T1 (Tetra) $m = 0$, $n = 1$; H1 (Hexa) $m = 1$, $n = 1$; and O1 (Octa) $m = 2$, $n = 1$. Where the uronic acid residue closest to the reducing end is iduronic acid and $R = SO_3^-$: D2 (Di), $m = n = 0$; T2 (Tetra) $m = 1$, $n = 0$; H2 (Hexa) $m = 1$, $n = 1$; O2 (Octa) $m = 1$, $n = 2$; D10 (Deca), $m = 1$, $n = 3$; D12 (Dodeca), $m = 1$, $n = 4$; D14 (Tetradeca), $m = 1$, $n = 5$; and D16 (Hexadeca), $m = 1$, $n = 6$. Synthetic heparin pentasaccharide P5. (C) Dermatan sulfate where the uronic acid residue is 95% iduronic acid and 5% glucuronic acid and dermatan sulfate oligosaccharides: DS2 (Di) $n = 0$; DS4 (Tetra) $n = 1$; DS6 (Hexa) $n = 2$; and DS8 (Octa) $n = 3$.

O2 is the minimum sized binding partner required for strong interaction with the apoE4 N-terminal domain and is consistent with prior studies implicating this domain in apoE-related neurotoxicity. Higher inhibition was observed for a heparin dodecasaccharide, but this oligomer resulted in a large number of floating and clumping cells in the assay. The IC_{50} values of heparin octasaccharides through hexadecasaccharides, ranged from 1.1 to 2.4 μM, only 5- to 10-fold higher than the IC_{50} of heparin. This suggests that heparin and heparin oligosaccharides may inhibit apoE peptide neurotoxicity through a similar binding mechanism, a possibility that will require additional studies. However, the fact that an octasaccharide is as effective at inhibiting toxicity as oligosaccharides with a higher number of residues suggests that larger oligosaccharides do not increase protective activity. The IC_{50} of dermatan sulfate oligosaccharides was approximately 500-fold higher than dermatan sulfate, indicating that dermatan sulfate may inhibit apoE neurotoxicity through a binding mechanism different from the one involving heparin. This mechanism could include multimeric binding instead of the 1:1 stoichiometry observed for apoE/heparin complexes (*48*). It is important to remember that the natural ligand of apoE is probably heparan sulfate. While investigating heparan sulfate oligosaccharides could give additional insights into the mechanism of inhibition, pure heparan sulfate oligosaccharides of comparable size cannot

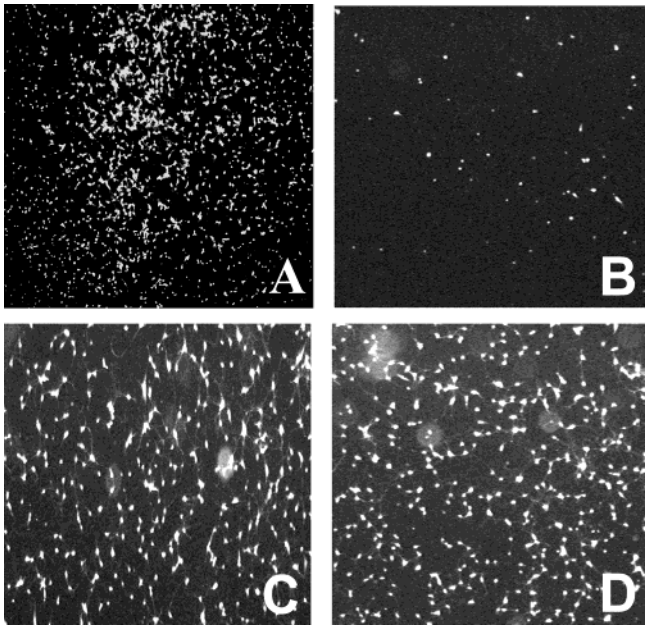

FIGURE 4: In vitro neuron death assay: (A) Dissociated sympathetic neurons were cultured for 20 h and then exposed to vehicle alone; (B) apoE peptide; (C) oligosaccharide O2; or (D) apoE peptide + O2. Surviving cells were labeled with a fluorescent dye that is only accumulated by living cells. The majority of neurons were killed by exposure to the peptide (B). The few remaining cells are primarily nonneuronal cells. O2 did not cause neuronal cell death (C) and protected against the neurotoxicity of the apoE peptide (D). All images were taken with a 4× objective. C and D are reproduced at approximately 2-fold greater magnification to demonstrate the integrity of neuronal morphology in the presence of the octasaccharide.

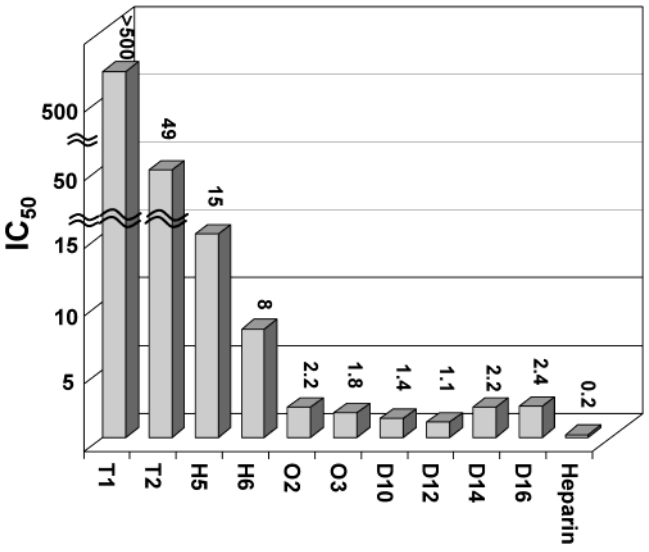

FIGURE 5: Neurotoxicity inhibition of heparin oligosaccharides. The bar graph indicates the IC_{50} (micromolar) for inhibition of apoE peptide toxicity by a series of oligosaccharides. The most effective inhibitors are oligosaccharides with eight or more saccharide residues.

be as readily prepared. Commercial heparan sulfates prepared from kidney or intestine have fewer sulfo groups (<1/disaccharide) than heparin (~2.7/disaccharide). However, it has been shown that heparan sulfate isolated from the brain or liver has a much higher content of sulfo groups, closely resembling heparin (*44*, *51*). Therefore, the physiological ligand of apoE may have a much higher degree of sulfonation than heparan sulfate from traditional sources and heparan sulfate oligosaccharides. Oligosaccharides generated from brain or liver heparan sulfate might more closely resemble the heparin oligosaccharides used in the current study than the more difficult to prepare heparan sulfate oligosaccharides. Future investigations will focus on the in vivo evaluation of the heparin octasaccharide O2, which displays a low IC_{50} of 1.8 μM and does not induce the floating and clumping of cells.

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