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Exploring the mechanism of β amyloid toxicity attenuation by multivalent sialic acid polymers through the use of mathematical models

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Abstract

β -Amyloid peptide (A β), the primary protein component in senile plaques associated with Alzheimer's disease (AD), has been implicated in neurotoxicity associated with AD. Previous studies have shown that the A β -neuronal membrane interaction plays a role in the mechanism of A β toxicity. More specifically, it is thought that A β interacts with ganglioside rich and sialic acid rich regions of cell surfaces. In light of such evidence, we have used a number of different sialic acid compounds of different valency or number of sialic acid moieties per molecule to attenuate A β toxicity in a cell culture model. In this work, we proposed various mathematical models of A β interaction with both the cell membrane and with the multivalent sialic acid compounds, designed to act as membrane mimics. These models allow us to explore the mechanism of action of this class of sialic acid membrane mimics in attenuating the toxicity of A β . The mathematical models, when compared with experimental data, facilitate the discrimination between different modes of action of these materials. Understanding the mechanism of action of A β toxicity inhibitors should provide insight into the design of the next generation of molecules that could be used to prevent A β toxicity associated with Alzheimer's disease.

Keywords

Alzheimer's disease; computational model; N-acetylneuraminic acid; ganglioside

Introduction

Alzheimer's disease (AD) is the leading cause of neurodegeneration in the United States, affecting approximately 5.2 million Americans in 2008, with an annual cost of care for these individuals estimated at approximately \$150 billion (Alzheimer's Association, 2008).

Alzheimer's disease is characterized by the presence of neurofibrillary tangles and amyloid plaques. The main protein component of the plaques is beta amyloid peptide (A β). It has been hypothesized by many that A β plays a causative role in the neurodegeneration associated with AD.

The mechanism by which A β causes neurotoxicity is the subject of much debate. Most believe that A β toxicity is linked to the formation of aggregated species, with the most toxic species being an intermediate between monomer and fibrils (Dahlgren et al., 2002; Hoshi et al.,

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2003; Kaye et al., 2004; Wang et al., 2002). Some believe that A β acts via association with the cell membrane (Whitson et al., 1994), to cause membrane depolarization (Blanchard et al., 2002), changes in membrane capacitance (Sokolov et al., 2006), membrane destabilization (Kilsdonk et al., 1995), pore formation (Arispe et al., 1993; Diaz et al., 2006), or free radical generation (Butterfield et al., 2002; Varadarajan et al., 2000). The first step in any of the above mechanisms of A β action on the cell would be A β binding to the cell membrane.

A number of investigators have suggested that A β binds to cell membranes through interaction with cell surface gangliosides or glycoproteins containing sialic acid (Ariga and Yu, 1999; Ariga et al., 2001b; Choo-Smith and Surewicz, 1997; Matsuzaki and Horikiri, 1999; Wakabayashi et al., 2005; Yanagisawa et al., 1995; Zha et al., 2004). In these studies, binding affinity of A β to the membrane was higher when multiple sialic acids were present, either because of clustering of gangliosides or because of the degree of sialylation of the gangliosides (Ariga et al., 2001a; Kakio et al., 2001; Kakio et al., 2002; Kakio et al., 2004). In an unrelated study, we showed that removal of cell surface sialic acids almost completely attenuated A β toxicity in a cell culture model (Wang et al., 2001). Based on this evidence, we synthesized a number of membrane mimicking, multivalent sialic acid polymers, which we then added to cells in culture to attenuate toxicity (Cowan et al., 2008; Patel et al., 2006; Patel et al., 2007). These materials also bound to A β with high affinity (on the order of 10^7 to 10^8 M $^{-1}$ association binding constants, compared to 10^6 – 10^7 M $^{-1}$ for sialic acid containing membranes to A β) (Cowan et al., 2008; Patel et al., 2006; Patel et al., 2007). While these materials have been successful at attenuating A β toxicity, the effectiveness of these materials in preventing A β toxicity displayed an unexpected dependence on the concentration of A β . At high concentrations of A β , the multivalent sialic acid polymers were less effective at attenuating A β toxicity, regardless of the concentration of sialic acid polymer added, even when results were normalized to account for the increase in cell toxicity at higher A β concentrations.

In this work, we develop a series of simple mathematical models based on different mechanisms of reactions of multivalent sialic acid polymers and A β with each other and with cells. We use the models to generate predictions of cell viability in the presence of A β and membrane mimicking multivalent sialic acid polymers. We compare each model to experimental data to evaluate the plausibility of the mechanism of action of the sialic acid polymers. Results from this analysis suggest that membrane mimicking sialic acid polymers do not simply compete with the cell surface for A β binding to attenuate toxicity. Electrostatic interactions between the polymers and cells or A β can not totally account for the observed behavior. The most plausible mechanism explored is that sialic acid polymers bind to A β , and alter the rate at which the A β interacts with the cell and induces the toxic effect. Our results highlight the role that mathematical modeling can play in understanding biological mechanisms and indicates both new experiments and new strategies that might be employed to better attenuate A β toxicity associated with disease.

Materials & Methods

Sialic acid polymers either made by conjugating N-acetylneuraminic acid to polyamidoamine (PAMAM) dendrimers or by photocrosslinking disialyl-lacto-N-tetraose (DSLNT) were prepared as previously described (Cowan et al., 2008; Patel et al., 2006; Patel et al., 2007). A β_{1-40} (>98% purity by HPLC and mass spectrometry) was purchased from Biosource International (Camarillo, CA). Human neuroblastoma SH-SY5Y, mouse neuroblastoma N2A, and rat pheochromocytoma PC12 cells were purchased from ATCC (Manassas, VA). Cell culture reagents were purchased from Gibco-Invitrogen (Grand Island, NY). Human recombinant nerve growth factor- β (NGF- β) and sialic acid (N-acetylneuraminic acid, NANA) were purchased from Sigma-Aldrich (St. Louis, MO). Propidium iodide (PI) was purchased

from BD Biosciences (San Jose, CA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

A β Solutions

1 mg of lyophilized A β was dissolved in 100 μ l DMSO. The stock was then diluted to different concentrations in minimal essential media (MEM) and aggregated with gentle mixing at 34 rpm for 24 hours. This method of aggregation consistently produced toxic aggregated species in our hands (Lee et al., 2007).

Cell Culture and Viability Assays

SH-SY5Y cells and N2A cells were cultured in a humidified 5% CO₂/air incubator at 37 °C in MEM, supplemented with 10% (vol/vol) fetal bovine serum, 100 U/ml penicillin, 100 μ g/ml streptomycin and 2.5 μ g/ml amphotericin B (Fungizone). SH-SY5Y cells were NGF differentiated prior to use in toxicity experiments by addition of 20 ng/ml NGF to cells for 5–7 days in 96-well plates.

Rat pheochromocytoma PC12 cells were cultured in RPMI medium supplemented with 10% (v/v) horse serum, 5% (v/v) fetal bovine serum, 3 mM L-glutamine, 100 units/ml penicillin, 100 g/ml streptomycin, and 2.5 g/ml amphotericin B in a 5% (v/v) CO₂/air environment at 37 °C. Cells under passage 20 were used in all experiments.

Propidium iodide (PI) was used to measure the viability of the cells in all experiments, using methods previously described (Cowan et al., 2008; Patel et al., 2006; Patel et al., 2007). In brief, cells were seeded in a 96-well plates at a density of 50,000 cells/well. 24 hours after seeding, medium was removed and replaced with medium containing A β . Sialic acid compounds were then added separately to cells. Cells were incubated for an additional 24 hours in a CO₂ incubator, at which time cells were stained with 3.3 μ M PI for 30 minutes. Fluorescence staining of cells was assessed using flow cytometry (FACSArray Bioanalyzer, Becton-Dickinson, Bedford, MA). Cells were excited with a 532 nm laser and fluorescence was detected using a 564–606 nm filter. Gating was done so as to obtain percentages of the total cell population that were viable. Normalized viability values were obtained by dividing the percentage of viable cells in the sample by that in the control samples with no A β or other agent. All A β concentrations reported were expressed as concentration of A β monomer originally present in the sample before aggregation. Significance of results was determined using a t test with $p < 0.05$, unless otherwise indicated.

Mathematical model development

We first posed a very simple mechanism for A β induced cell death, that A β binds to a site (S) on the cell membrane, forming a complex (A β -S), with an equilibrium association constant, K_S . The presence of the complex on the cell membrane resulted in cell death via some undetermined mechanism with a rate of k_1 . This mechanism is described by reactions (1) and (2).

Differential and algebraic equations were written to describe the reactions associated with A β induced cell toxicity (see appendix).

In our base model along with all subsequent models, we used as a model input the concentration of A β expressed per monomer unit. While it is well accepted that aggregated A β species are responsible for toxicity, there is some uncertainty about the size of the toxic A β species and its relative concentration in a mixture of aggregated species (Lambert et al., 2001, Deshpande et al., 2006, Hoshi et al, 2004). Given the complexity of A β aggregation kinetics (Murphy 2007), we believed it would be unrealistic with the data available to model the concentration of toxic A β species. Instead, we made the implicit assumption that in samples of A β aggregated in a similar manner, the concentration of toxic species would be directly proportional to the initial concentration of A β monomer used.

We fit the base model to experimental data of cell viability as a function of A β concentration and time to obtain constants K_s and k_1 . We also obtained an estimate for the number of binding sites, S_0 , per cell. Fitting was done using a non-linear least squares regression algorithm programmed in MATLAB that took advantage of a built in optimization function (fminsearch) that utilized a simplex search method.

Once a satisfactory fit of experimental data for A β induced cell toxicity was obtained, we then posed models for the potential interaction of sialic acid containing molecules with A β that could lead to toxicity attenuation.

Model 1: Competitive binding of mimic to A β

We first proposed that A β bound to sialic acid containing molecules, which we hoped, mimicked the sites on the cell surface to which A β bound, via a competitive mechanism. We refer to these molecules as mimics (M). As shown in reaction (3), A β binds to a mimic forming an A β -mimic complex (A β -M) with an equilibrium association constant of K_M , effectively reducing the concentration of free A β that can react with the cell surface sites (S), thus killing cells (as described by reactions (1) and (2)). A β -mimic complexes are non-toxic and prevent A β from binding to cell surface sites.

Model 2: Noncompetitive binding of mimic to A β

If A β bound to mimics in a different orientation than A β bound to the cell surface, then A β would be able to bind to both mimics and sites on the cell surface simultaneously. To capture this possibility, we proposed that the A β -mimic complex could still bind to the cell surface at a site, S, forming an A β -mimic-cell surface site complex (A β -M-S) described by reaction (4). Analogously, the A β -cell surface site complex can bind to a mimic forming the same A β -mimic-cell surface site complex, described by reaction (5). Equilibrium association constants for reactions (4) and (5) are K_4 and K_5 , respectively. We assumed that the A β -M-S complex was non-toxic towards cells. However, the A β -S complex would still be toxic towards the cell if no mimic was present.

Model 3: Toxic A β -M Complex

As a variation of model 2, we assumed that A β could bind to a mimic, resulting in a complex (A β -M) which could then bind to a cell surface site (A β -M-S), or that a mimic could bind to an A β -cell surface site complex (A β -S) to form a A β -mimic-surface site complex (A β -M-S), as described by reactions (4) and (5). However, we assumed that the A β -mimic-surface site complex was toxic to cells, but less toxic than the A β -surface site complex.

Model 4: Multiple mimics bind to A β

We explored the possibility that multiple mimics could bind to a single molecule of A β to form complexes (A β -M₂) as shown in reaction (7). The A β -M₂ could either be inert (not interacting with the cell) analogous to competitive model 1, or interact with cells in the same fashion as A β -mimic complexes in models 2 and 3. This second possibility is described by reactions (8) and (9).

Model 5: Multiple A β bind to a single mimic

We allowed for the possibility that multiple A β could bind to a single mimic resulting in the complex (A β ₂-M), as shown by reaction (10).

Model 6: Electrostatic interactions

As a final possibility, we explored the possibility that sialic acid mimics did not bind directly to A β at all, but altered the reaction rate of A β with the cell surface indirectly. All the multivalent sialic acid materials used in this work contained multiple charges that could effectively shield the electrostatic interaction between A β and the cell surface. We proposed that the sialic acid mimics altered the binding of A β with the cell surface as a function of the ionic strength of the medium, which was a function of the concentration of multivalent sialic acid mimics as described by equation (11).

$$\log_{10} \frac{K_s}{K_{S_0}} = 1.02 \cdot z_A \cdot z_B \cdot I^{\frac{1}{2}} \quad (11)$$

where I is the medium ionic strength in units of mol/dm³, K_S is the binding constant of A β with the cell surface as described by reaction (1) at ionic strength I, K_{S0} is the binding constant

at zero ionic strength, and z_A and z_B are the charge on $A\beta$ and the cell surface, respectively. Ionic strength of the culture medium was estimated as:

$$I=0.5(0.320+z_{\text{Mimic}}^2 C_M) \quad (13)$$

where C_M is the concentration of sialic mimic, z_{Mimic} is the charge on the mimic, and 0.320 represents the sum of the ions in culture medium multiplied by the square of their charge in units of mol/dm^3 . This formulation of the effect of ion strength on equilibrium constants is taken from Laidler (1987).

Results and Discussion

We and others have found that $A\beta$ binds with high affinity to clustered sialic acids on the surface of cells and artificial biomolecules (Cowan et al., 2008; Kakio et al., 2002; Patel et al., 2006; Patel et al., 2007; Wang et al., 2001). We have also shown that removal of cell surface sialic acids with neuraminidase is able to completely attenuate $A\beta$ toxicity *in vitro* (Wang et al., 2001). Based on these observations, we hypothesized that $A\beta$ binding to cell surface sialic acids was an early step in $A\beta$ induced cell toxicity. To test this idea, we developed different types of multivalent sialic acid materials which we hoped would competitively inhibit $A\beta$ binding to the cell surface, by providing a soluble binding site for $A\beta$, and thus attenuate $A\beta$ toxicity (Cowan et al., 2008; Patel et al., 2006; Patel et al., 2007). Figure 1 shows the toxicity results of simultaneous incubation of cells with varying doses of $A\beta$ and mimic molecules in the form of soluble sialic acid, sialic acid conjugated generation 2.0 PAMAM dendrimers, and photocrosslinked DSLNT. As can be seen, orders of magnitude lesser quantities/doses ($1\mu\text{M}$) of the multivalent sialic acid clusters were needed to attenuate $A\beta$ induced cell death, compared to soluble sialic acid (1mM). The decrease in concentration of multivalent sialic acid compounds needed to prevent toxicity as compared to monovalent soluble sialic acid is not surprising since it is well established that multivalency can increase binding affinity of ligand to receptors (Kumar et al., 2006; Lees et al., 1994; Polizzotti and Kiick, 2006; Prieto et al., 2006; Woller and Cloninger, 2002). While these materials were able to attenuate $A\beta$ toxicity, their efficacy to do so was limited to lower $A\beta$ concentrations (Figure 1). The trend in was observed regardless of the cell line employed. This surprising result suggests that the sialic acid mimics may interact with $A\beta$ and cell surfaces via more complicated interactions other than merely competing with the cell surface for $A\beta$ binding. The concentration at which sialic acid compounds attenuated $A\beta$ toxicity did not appear to change with concentration of $A\beta$, however, the maximum increase in viability achieved with sialic acid materials decreased at higher $A\beta$ concentrations.

To explore the mechanism by which sialic acid compounds interacted with $A\beta$ to attenuate toxicity, we developed a series of simple mathematical models which capture the following interactions or phenomenon that ultimately lead to toxicity: $A\beta$ binding to sites on the cell surface to form $A\beta$ -cell surface site complexes, the formation of which leads to cell death; $A\beta$ binding to sialic acid mimics to form $A\beta$ -mimic complexes, that may in turn interact with cell surface sites and cause cell death, or merely reduce the concentration of free/soluble $A\beta$ that can bind to cell surface sites to cause cell death.

As our base model, we assumed that $A\beta$ binds to sites on the cell surface to form $A\beta$ -cell surface site complexes, the formation of which leads to irreversible cell death (reactions 1 and 2). In Figure 2, we show the model estimates of cell viability upon exposure to $A\beta$ from this base model. Constants used in the model are found in Table 1. Using this base model of $A\beta$ interaction with cells in the absence of mimics, we can reasonably capture trends in experimental cell viability data as a function of time of $A\beta$ exposure and $A\beta$ concentration

(experimental data in Figure 2 adapted from Patel and Good, 2007 with permission from Elsevier). The model was used to fit A β toxicity data in SY5Y cells, however, SY5Y, PC12 and N2A cells were used in experiments to examine the ability of sialic acid materials to attenuate A β toxicity. The three different cell lines used in experiments had different susceptibilities to A β toxicity, with PC12 cells being most susceptible to A β toxicity, while differentiated SY5Y cells and N2A cells had comparable susceptibility to A β toxicity. In unpublished experiments, we have found that susceptibility to A β toxicity correlates with cell surface sialic acid and ganglioside levels, which would affect S_0 in our model.

The binding constant of A β to the cell surface site (K_S) estimated from the fit of the model to the experimental data, $1.25 \times 10^5 \text{ M}^{-1}$, is comparable to inhibition constants estimated from A β blockade of a potassium channel (Good et al., 1996) and from toxicity data (Wang et al., 2002). The estimated initial number of binding sites on the cell surface, S_0 , is approximately 70 billion sites per cell. This number of binding sites per cell is significantly greater than the estimated number of GM1 molecules per cell, at roughly 30 to 60 thousand molecules per cell for SY5Y and N2A cells respectively (Yang et al., unpublished results). However, there are many other sources of sialic acid per cell (Allende and Proia, 2002; Bork et al., 2005; Ryll et al., 2000), along with a number of postulated specific and non-specific binding sites per cell to which A β could bind (Mruthinti et al., 2006; Verdier et al., 2004). In addition, K_S , k_1 , and S_0 co-varied, in that estimates of one constant were dependent upon estimates of the other constants. Finally, we assumed that the A β concentration expressed per monomer unit represents the concentration of toxic species. However, it is well accepted that the toxic A β species is some oligomer of some size between about 10 monomer units, as might occur in an ADDL (Hepler et al., 2006; Lambert et al., 2001), and 100 monomer units or more, as might be estimated for a spherical intermediate described by others (Chimon et al., 2007; Hoshi, 2004; Lee et al., 2007). We typically prepared A β for toxicity assays via a protocol in which fibrils are formed, which might contain close between ten thousand and one million monomers. Thus we likely overestimated the concentration of toxic A β species by at least a factor of 1000 (given that the species in solution were most likely a combination of toxic oligomer, less toxic fibril, and nontoxic monomer). If we had overestimated the concentration of toxic A β species by a factor of 1000, then, for A β , cell surface sites, and complexes to change with the same dynamics, we would have to have overestimated the initial number of sites on the cell surface for A β binding by a factor of 1000 as well. Using simple geometry arguments, assuming an A β oligomer is 10 nm in diameter and a cell is 20 μm in diameter, at most 4 million oligomers could bind to a cell if they covered the entire surface. Thus, even 60 million A β binding sites per cell is an overestimate, and most likely indicates that the A β in solution contains a heterogeneous mixture of toxic oligomer and fibril along with other non-toxic species.

We explored the possibility that more than one molecule of A β was needed to bind to a site on the cell surface in order to form a complex on the cell surface that would then lead to loss of cell viability (model predictions not shown). Such scenarios, however, did not lead to improved correlation of model predictions compared to experimental data thus were rejected in favor of the simplest model proposed.

Using our base model for A β induced cell toxicity, we then added additional equations to describe the competitive binding of A β to sialic acid mimics in solution (Model 1, described by reactions (1), (2) and (3)). The physical interpretation of the model is that A β binds to the mimics via the same binding site or amino acid residues as it does to the cell surface. Thus, once A β is bound to a mimic, it will be unavailable to bind to the cell surface and induce toxicity. Predictions of the competitive inhibition model are shown in Figure 3. As shown, this model suggests that if mimics are added at a high enough concentration, A β toxicity should be totally attenuated. Moreover, the maximal increase in viability achieved is predicted to be greater for higher A β concentrations. These 2 observations are not consistent with our

experimental data for any of the sialic acid compounds shown in Figure 1. Based on the comparison of predictions from model 1 and our experimental data, we conclude that model 1, competitive binding of A β to sialic acid mimics does not capture the trends seen in our experimental data and therefore probably does not represent the mechanism of action of these materials.

It is possible that model 1 did not capture trends observed in experimental data because the constants used in the model were inappropriate. We believe that K_{M1} , the binding constant of A β to the mimics, is reasonable because it is within the range of equilibrium binding constants we have measured experimentally for these materials (Cowan et al., 2008; Patel et al., 2006; Patel et al., 2007; Wang et al., 2001). Additionally, the sialic acid materials used in our experiments had in binding affinities for A β in the range of 10^4 M^{-1} to 10^8 M^{-1} , thus suggesting the trend in behavior was not dependent upon binding affinity of the materials but on the general mechanism of action of these materials.

Another explanation for the discrepancy between our experimental results and model predictions is that we did not use high enough concentrations of mimic to observe the total toxicity attenuation predicted at high concentration mimics. For some of the multivalent sialic acid materials we have synthesized, especially sialic acid conjugated dendrimers, we have observed cytotoxicity of the materials at high concentrations (about 50 μM for generation 2 PAMAM dendrimer (Patel et al., 2007)). We did not observe cytotoxicity at concentrations below 100 μM with the photocrosslinked DSLNT (Cowan et al., 2008). In Figure 1, we see a clear plateau in toxicity attenuation at concentrations below the toxic concentrations of the multivalent sialic acid materials. We conclude, therefore, that the most likely explanation for the discrepancy between experimental results and model predictions is not the toxicity of sialic acid compounds, but that the sialic acid mimics do not interact with A β via a competitive mechanism.

We next proposed a partially non-competitive inhibition model in which A β binds to mimics but via a different site than that used for A β binding to the cell surface. In this model, A β would be able to bind to both mimics and the cell surface sites simultaneously. Reminiscent of enzyme kinetics, we would expect that toxicity attenuation would then change with the amount of A β added under these conditions (Shuler and Kargie, 2002). The noncompetitive model (model 2) is described by reactions (1), (2), (3), (4), and (5). The new species formed in this model, the A β -mimic-site complex (A β -M-S), is assumed to be non-toxic to cells. Predictions from this model are shown in Figure 4. Compared to the competitive model, the predictions from the non-competitive model did not better capture the qualitative trends observed in experimental data. The values for equilibrium binding constants used to generate viability predictions shown in Figure 4 are included in Table 1. We also examined viability predictions for this model when we changed the equilibrium constants K_4 and K_5 by two orders of magnitude in each direction (model predictions not shown). While changes in parameters shifted the positions of the predicted viability curves, the qualitative trends of the model predictions remained unchanged. Thus, we rejected the non-competitive model as a representation that captures the mechanism of action of sialic acid mimics with A β that leads to toxicity attenuation.

We next allowed for the possibility that the A β -mimic-cell surface site complex (A β -M-S) could induce cell toxicity with a rate constant k_3 . This third model is described by reactions (1), (2), (3), (4), (5), and (6). The physical interpretation of this model is that the A β -mimic complex can still bind to the cell and kill the cell, but the presence of the mimic bound to A β reduces the toxicity of A β . Predictions of cell viability as a function of mimic concentration for the third model are shown in Figure 5. As seen in the figure, based on this model, A β toxicity attenuation was not predicted to be complete at higher concentrations of A β . Toxicity

attenuation by the mimics was predicted to decrease as the concentration of A β was increased. This is consistent with the trends in experimental viability data observed in Figure 1, where toxicity attenuation by sialic acid containing compounds decreases at elevated concentrations of A β . However, viability predictions based on model 3 suggest that the concentration of mimic needed to prevent A β toxicity increases at higher concentrations of A β , a trend not observed in the experimental data.

While predictions from model 3 do not perfectly match our experimental data, some key trends were observed. We think the mechanism of action of sialic acid mimics on A β may be captured by model 3. The toxic A β species is postulated to be made up of many A β monomers, and may have a diameter on the order of 10 to 30 nm (Hoshi, 2004; Lee et al., 2007; Takano et al., 2006; Wang et al., 2002). The diameter of the sialic acid mimics used in our experiments is on the order of 3 to 15 nm. Therefore it is unlikely that any one mimic could completely block all residues on A β that participate in binding with the cell surface. It is also quite probable that the arrangement of sialic acids in mimics, either in the sialic acid modified PAMAM dendrimers or in the photopolymerized oligosaccharides, is significantly different than the arrangement of sialic acids on the cell surface, making it improbable that the mimics would effectively or completely compete for A β binding to the cell surface.

A number of researchers have proposed that the ability of A β to undergo a conformational change once bound to the cell may be a key factor in the mechanism of A β toxicity (Lee et al., 2007; Kremer et al., 2000; Lau et al., 2006; Matsuzaki and Horikiri, 1999). For example, if A β were to form a pore in the membrane upon binding, the peptide would have to rearrange from the spherical form observed in solution (Micelli et al., 2004; Mirzabekov et al., 1996; Yoshiike et al., 2007b). The presence of a mimic bound to the A β surface might alter the ability of the peptide to undergo the structural rearrangement necessary for toxicity, thus reducing the toxicity of the A β -mimic complexes consistent with model 3.

Finally, we suggest that model 3 might be plausible in light of seemingly contradictory evidence, some of it from our own lab (Wang et al., 2001), because cell surface sialic acids have multiple functions. Cell surface sialic acids can serve as a receptor for A β or other ligand binding and can serve as a part of a complex for signal transduction. The sialic acid mimics developed in our lab would likely only affect ligand binding, and not other possible cell surface sialic acid functions.

We further modified the mathematical model to allow for the possibility that more than one molecule of A β bound per mimic or more than one mimic bound per A β , as described by reactions (7)–(10). While these modifications of the models led to changes in the slope of the viability curve at low mimic concentrations, they did not lead to qualitatively different viability predictions as a function of A β concentration (model predictions not shown).

The last mechanism we evaluated took into consideration the possible electrostatic interactions between A β , the cell, and sialic acid mimics. There have been reports by others that electrostatic forces contribute to the A β interaction with the cell membrane (Hertel et al., 1997; Terzi et al., 1994; Yoshiike et al., 2007b). It has been suggested that either the lysines or histidines on the toxic A β species interact with a negatively charged group on the cell surface, possibly sialic acid (Calamai et al., 2006; Yun et al., 2007). Thus the presence of other charged species in solution could shield the electrostatic attraction. Consistent with this hypothesis, we have seen that PAMAM dendrimers, generation 2 and 3, with 16 and 32 positive charges on the surface, respectively, but without sialic acid modification, have modestly but significantly attenuated A β toxicity when added to cells at micromolar concentrations (Patel et al., 2006).

In this model, we assumed that the sialic acid mimics did not bind to A β , but simply altered the rate of A β binding to the cell surface. We use the formulation described by equation (11)

to predict the effect of charged molecules on the equilibrium binding constant for A β with the site on the cell surface. The results of viability predictions as a function of mimic concentration based on this model are shown in Figure 6. Modest toxicity attenuation was predicted at all A β concentrations. Toxicity attenuation appeared to be only a weak function of A β concentration, and did not saturate at any range of parameters examined. While these predictions do not capture the qualitative features of the experimental data shown in Figure 1, they indicate that electrostatic shielding may contribute to the toxicity attenuation observed when cells are treated with highly charged polymers.

When we estimated the effect of the ionic strength of the sialic acid mimic on K_S and cell viability, we took into account that the cell culture medium had a finite ionic strength (about 160 mM) without addition of the mimic. In order to estimate the effects of ionic strength on equilibrium constants, we needed an estimate of the product of the charge of the toxic A β molecule and the cell surface site. For the viability predictions shown in Figure 6, we assumed the product of the charge on the toxic A β and the cell surface site was -10 . There was little experimental basis for this assumption. In fact, while some researchers suggest that there is an attractive electrostatic contribution to the interaction of A β with the cell surface (Calamai et al., 2006; Yun et al., 2007), and have observed clusters of positive charges on the A β fibril surface (Yoshiike et al., 2007a), the charge on an A β monomer is generally taken to be negative (on the order of -0.5 to -1) (Chrambach et al., 2000; Lewczuk et al., 2004), the charge on a toxic oligomer would be at least 10 to 100 times higher (Petkova et al., 2006), and the charge on the cell surface is generally accepted to be negative (Calamai et al., 2006; Yun et al., 2007). Thus while there may be a local electrostatic attraction when the A β is in close proximity to the cell surface, this electrostatic attraction diminishes with growing distance between the A β and cell surfaces.

To estimate the ionic strength of sialic acid polymer solutions, we took the charge on a polymer to be 16. For PAMAM dendrimers, the net charge on the sialic acid modified dendrimer might be close to 16, depending upon the degree of sialic acid modification, however, this is likely to be an overestimate for our photocrosslinked oligosaccharide polymers. Based on our estimates of the polymer molecular weight, we estimated charge to be between 6 and 10. If the charge on the polymer was taken as 6, then at physiological ionic strength (as used in our experiments), we predicted that the polymers would increase cell viability in the presence of A β via electrostatic shielding by at most 1 or 2 percent at the highest concentrations of polymers used. At physiological ionic strengths, electrostatic effects are likely to play a role in sialic acid polymer toxicity attenuation of A β only for very highly charged polymers.

It is possible that sialic acid mimics attenuate A β toxicity by binding directly to cells instead of either binding to A β or shielding electrostatic effects. This is certainly likely for the case of positively charged PAMAM dendrimers (Figure 1B), as we and others have observed high intrinsic toxicity of the higher generation dendrimers (Patel et al., 2006). However, we feel that direct binding of sialic acid mimics is less likely in the case of photopolymerized oligosaccharides (Figure 1C), as the only charge on these polymers is the negative charge associated with the sialic acid. Thus, we did not explore this mechanism further.

In summary, the model that best qualitatively captured the trends in viability observed when cells were treated with both A β and sialic acid containing molecules was model 3 (Figure 5), in which the sialic acid mimic bound to A β , the A β -mimic complex was still toxic to the cell, but with reduced toxicity compared to A β alone. In addition model 6 (Figure 6), in which sialic acid mimics did not bind A β , but simply shielded the electrostatic interaction between A β and the cell surface indicates that it may be possible to attenuate the toxicity of A β using a highly charged polyelectrolyte. It is possible that both mechanisms contribute to the A β toxicity attenuation observed when cells are treated with multivalent sialic acid compounds. Model 3

and model 6 were combined such that we could examine cell viability predictions when we considered the effects of a toxic A β -mimic complex and the electrostatic shielding effects of the mimic. Predictions generated by the combined model did not differ qualitatively from those obtained with model 3 alone (data not shown) suggesting that electrostatic effects are less important than the effects of forming the A β -mimic complex on the ability of multivalent sialic acid polymers to attenuate A β toxicity.

We and others have previously directly measured the equilibrium constant for A β binding to different sialic acid containing molecules including those described in this work. Binding constants have ranged from 10^4 M^{-1} for monovalent soluble sialic acid (Yoo, 2002), 10^6 to 10^7 M^{-1} for mono and divalent sialic acid containing gangliosides (Ariga et al., 2001b), and 10^7 to 10^8 M^{-1} for trivalent sialic acid polymers (Cowan et al., 2008; Patel et al., 2006; Patel et al., 2007). If we assume that the free energy of binding of each sialic acid to A β in the multivalent polymers is additive, and that the backbone of the polymer contributes to the total free energy of binding, we can estimate the free energy contribution of binding for each sialic acid and the backbone. Based on this simple analysis, the free energy associated with each sialic acid binding to A β is on the order of 2.7 kcal/mol, which is along the same order as an electrostatic interaction (Albeck et al., 2000). The free energy associated with the sialic acid polymer backbone interacting with A β is on the order of 1.8 kcal/mol or 3 hydrophobic or hydrogen bonding interactions (Ross and Rekharsky, 1996). This analysis suggests that our estimates of A β binding to sialic acid mimics are reasonable and rule out the possibility of any mechanism of action of mimics in which binding to A β is not involved.

Having insights into the mechanism of action of molecules like the multivalent sialic acid polymers described in this work that attenuate A β toxicity will aid in the design of the next generation molecules for A β toxicity prevention. Our analysis suggests that sialic acid polymers bind to A β , and reduce but do not eliminate its toxicity. It may be fruitful to design new molecules like our sialic acid polymers which bind and sequester A β , but do not cross the blood brain barrier. Such molecules may be able to reduce the total amount of A β in the cortex, without contributing to cell toxicity.

If the mode of action of our sialic acid polymers is their ability to stabilize the A β structure such that it does not undergo the structural rearrangement necessary for the peptide to form a pore in the membrane or some other feature that induces cell toxicity, then it may be advantageous to design molecules which stabilize the A β oligomer. These molecules would not have to mimic the cell membrane surface. Their only design criteria would be the prevention of the peptide rearrangement that led to toxicity.

The reduction of electrostatic interactions between A β and the cell surface may contribute at least partially to the mode of action of sialic acid polymers. A number of polyelectrolytes are known to interact with A β , and alter its aggregation and/or toxicity (Calamai et al., 2006; Terzi et al., 1994). Investigating new ways to disrupt electrostatic interactions between A β and cell membrane components may be useful in designing new means to prevent cytotoxicity associated with A β .

The mathematical modeling analysis performed in this work can not be used to determine with certainty the mechanism by which A β interacts with multivalent sialic acid polymers. However, the analysis is useful in eliminating mechanisms of action that do not fit experimental data. In addition, the modeling and analysis are useful in identifying new hypotheses about the mode of action of the sialic acid polymers and the types of reactions that may be needed to effectively prevent A β toxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Toxicity attenuation of A β by soluble sialic acid (A), sialic acid conjugated dendrimers (B), and photopolymerized sialic acid containing oligosaccharides (C), as a function of the concentration of sialic acid containing molecule. (A) Viability was tested in PC12 cells at A β concentrations of 5 μ M (squares), 10 μ M (triangles), 15 μ M (circles), and 20 μ M (diamonds). (B) Viability was tested in differentiated SH-SY5Y cells at A β concentrations of 20 μ M (circles), 50 μ M (diamonds), and 75 μ M (triangles). (C) Viability was tested in N2A cells at A β concentrations of 20 μ M (diamonds) and 40 μ M (squares). Cells were treated for 24 hours with A β and sialic acid containing molecule, after which viability was measured with a PI assay. The fraction of viable cells, or cell viability of the sample divided by cell viability

of an untreated control population, is plotted. $n=3$ or more independent measurements. Lines represent the best fit saturation curve to the data, where the saturation curve is given by

$V = V_0 + \frac{(1 - V_0) \cdot S}{K + S}$, where V is the fraction of viable cells, V_0 is the fraction of viable cells treated with $A\beta$ but not treated with sialic acid or polymer, S is the concentration of sialic acid or polymer, and K is a saturation constant. Viability data upon treatment with photopolymerized sialic acid containing oligosaccharide (Figure 1 C) is adapted from Cowan et al., 2008, with permission from Elsevier.

Figure 2.

Fit of base model of A β induced cell toxicity as described by reactions (1) and (2) to experimental data of cell viability as a function of time of exposure to 100 μ M A β (A) and as a function of concentration of A β after 24 hours exposure to peptide (B). Experimental data shown as solid circles. Model fit shown as dashed line. Viability tested in differentiated SH-SY5Y cells using PI assay. $n = 3$ or more. Experimental data adapted from Patel and Good, 2007, with permission from Elsevier.

Figure 3.

Predictions of cell viability as a function of sialic acid containing mimic concentration for 5 different concentrations of Aβ: 10μM (thick solid line), 20μM (dash-dot-dash line), 40μM (dotted line), 50μM (solid line), and 75μM (thick dotted line). Model 1, the competitive inhibition model, was used in order to obtain cell viability predictions. Predictions were for 24 hour exposure of cells to Aβ and mimics.

Figure 4.

Predictions of cell viability as a function of sialic acid containing mimic concentration for 5 different concentrations of Aβ: 10μM (thick solid line), 20μM (dash-dot-dash line), 40μM (dotted line), 50μM (solid line), and 75μM (thick dotted line). Model 2, the noncompetitive inhibition model, was used in order to obtain cell viability predictions.

Figure 5.

Predictions of cell viability as a function of sialic acid containing mimic concentration for 5 different concentrations of A β : 10 μ M (thick solid line), 20 μ M (dash-dot-dash line), 40 μ M (dotted line), 50 μ M (solid line), and 75 μ M (thick dotted line). Model 3, the noncompetitive inhibition model with A β -M-S being a toxic species, was used in order to obtain cell viability predictions.

Figure 6.

Predictions of cell viability as a function of sialic acid containing mimic concentration for 5 different concentrations of A β : 10 μ M (thick solid line), 20 μ M (dash-dot-dash line), 40 μ M (dotted line), 50 μ M (solid line), and 75 μ M (thick dotted line). Model 6, the electrostatic interaction model, in which the rate of A β binding to a cell surface site is reduced as a function of the ionic strength of the mimics, was used in order to obtain cell viability predictions.

Table 1

Parameters used in model development.

Constant	Reaction	Value
K_S	1	$1.25 \times 10^5 \text{ M}^{-1}$
S_0	1	$1.1 \times 10^{-4} \text{ M}$
k_1	2	$3.2 \times 10^{-4} \text{ hr}^{-1}$
K_{M1}	3	10^8 M^{-1}
K_4	4	$1.25 \times 10^5 \text{ M}^{-1}$
K_5	5	10^8 M^{-1}
k_3	6	$1.6 \times 10^{-4} \text{ hr}^{-1}$
$z_A z_B$	13	-10
z_{Mimic}	13	16
K_{S0}	13	$1.5 \times 10^9 \text{ M}^{-1}$