Title: INHIBITION OF BETA AMYLOID BINDING TO GLYCOSAMINOGLYCANS FOR TREATMENT OF ALZHEIMER'S DISEASE

Abstract

The present invention relates to compounds that inhibit the binding of glycosaminoglycans and proteoglycans to beta amyloid peptide (Aβ, Aβ), and to compounds that inhibit Aβ activation of complement. In one aspect, the compound is a peptide having an amino acid sequence X-X-N-X, in which X is an amino acid with a cationic side chain and N is a neutral amino acid. In another aspect, the compound is a peptide having an amino acid sequence X-X-N-X-Z, in which X is an amino acid with a cationic side chain, N is a neutral amino acid and Z is a neutral amino acid. In yet another aspect, the compound is peptide having an amino acid sequence X1-N-X2-X3, in which at least two of X1, X2, and X3 are independently an amino acid with an anionic side chain and the third X is an amino acid with an anionic side chain or a neutral amino acid and N is independently a neutral amino acid. In yet a further aspect, the compound is an anionic disaccharide. The invention also relates to a method for treating Alzheimer’s disease comprising administering a therapeutically effective amount of a compound as described above to a subject suffering from Alzheimer’s disease. Pharmaceutical compositions are also provided.
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INHIBITION OF BETA AMYLOID BINDING TO GLYCOSAMINOGLYCANs FOR TREATMENT OF ALZHEIMER'S DISEASE

1. FIELD OF THE INVENTION

The present invention relates to compounds that inhibit the binding of glycosaminoglycans and/or proteoglycans to beta amyloid peptide (A(beta); Aβ), and to compounds that inhibit A(beta) activation of complement. The invention further relates to treatment of Alzheimer's disease by administration of the inhibitory compounds.

2. BACKGROUND OF THE INVENTION

A pathological hallmark of Alzheimer's disease (AD) is the presence of senile (neuritic) plaques within the cortex, hippocampus and certain subcortical nuclei of the brain (Terry and Katzman, 1983, in Neurology of Aging, Katzman and Terry, eds. F.A. Davis, p. 51; Terry et al., 1981, Ann. Neurol. 10:184-192). The most well-studied component of these plaques is βA4 or beta amyloid peptide (Aβ), a 40-43 residue peptide that is derived from the amyloid precursor protein (APP) (Glenner, 1984, Biochem. Biophys. Res. Comm. 120:885-890; Glenner, 1988, Cell 52:307-308; Mori et al. 1992, J. Biol. Chem. 267:17082-17086). It has been proposed that increased production of Aβ and its eventual deposition into insoluble plaques is a causative event in the etiology of AD. Aβ in insoluble plaques appears to activate the classical pathway complement (C) cascade without immunoglobulin mediation (Rogers et al., 1992, Res. Immunol. 143(6):624-630; Rogers et al., 1992, Proc. Natl. Acad. Sci. USA 89:10016-10020).
2.1. INTERACTION OF Aβ AND PROTEOGLYCANS

Aβ, while necessary, may not be sufficient in itself to induce the neuropathology of AD (Frederickson, 1991, Neurobiol. Aging 13:239-253). This concept receives further support from the recent observations that Aβ is produced normally by a variety of cell types (Haass et al., 1992, Nature 359:322-327; Shoji et al., 1992, Science 258:126-129). While Aβ is a major component of AD plaques, many other macromolecules have been identified within these structures, including dermatan sulfate proteoglycan (Snow et al., 1992, J. Histochem. Cytochem. 40:105-113) and heparan sulfate proteoglycan (HSPG) (Snow et al., 1988, Am. J. Pathol. 133:456-463). The latter appears to be found throughout the plaque core, and recent studies indicate that HSPG binds both APP (Narindrasorasak et al., 1991, J. Biol. Chem. 266:12878-12883) and Aβ (Snow et al., 1991, Soc. Neurosci. Abstr. 17:1106) with high affinity. The significance of proteoglycan localization within senile plaques is not known. It had been previously postulated that proteoglycan binding to amyloid may induce the latter to be deposited as insoluble fibrils, and that such interaction may also make the amyloid resistant to proteolytic degradation (Frederickson, 1991, Neurobiol. Aging 13:239-253).

The aforementioned interactions of HSPG with Aβ and its precursor appear to be mediated in part by the core protein of the proteoglycan (Narindrasorasak et al., 1991, J. Biol. Chem. 266:12878-12883; Snow et al., 1991, Soc. Neurosci. Abstr. 17:1106). However, the glycosaminoglycan chains of the proteoglycan may also be involved in the association with APP and Aβ since binding of both are diminished in the presence of heparin (Narindrasorasak et al., 1991, J. Biol.
2.2. COMPLEMENT ACTIVATION ASSOCIATED WITH ALZHEIMER'S DISEASE

The classical complement pathway is an immune-defense mechanism that results in activated molecules that can initiate inflammation, phagocytosis and cell lysis. There are 11 distinct protein components (C1q,r,s, and C2-C9) in the classical complement system, and activation of the cascade results in the proteolytic conversion of many of these molecules into forms that subsequently trigger further amplification of the pathway. An ultimate result of complement activation is the formation of "membrane attack complexes" (MACs) composed of C5-C9 that insert into cellular membranes and form large transmembrane channels that can lyse or damage foreign cells. A side-effect of complement activation can be "bystander" damage due to MAC attachment to nearby cells that are not specifically targeted for destruction.

The usual biological signal that causes activation of the classical complement pathway is the binding of specific immunoglobulins to antigens on foreign cells. If two immunoglobulin (Ig) G molecules are within close proximity on a cellular membrane, complement component C1q binds the Fc portions of the IgG proteins, with resulting activation of C1 and subsequent induction of the complement cascade. Prior research has shown that the binding of a single IgG to C1q is insufficient to cause activation, and it appears that two or more IgG molecules must bind to the multivalent C1q protein.

In Alzheimer's disease (AD) brain, there is evidence of activated complement components associated
with the senile plaques and dystrophic neurites that are the hallmark pathological features of this devastating disease (see, e.g., Johnson et al., 1992, Neurobiol. Aging 13:641-648). The presence of complement proteins in the AD brain is surprising, since normal brain is essentially devoid of complement. Previous reports of MAC localization to dystrophic neurites would suggest that the neuronal damage seen in AD results at least in part from "bystander" effects of complement activation.

Rogers and co-workers have shown directly in vitro and indirectly in situ that β-amyloid deposited in amyloid plaques activates complement (Rogers et al., 1992, Proc. Natl. Acad. Sci. USA 89:10016-10020). In particular, Rogers et al. showed that C1q immunoreactivity colocalizes with Aβ containing AD pathological structures and not immunoglobulins in the brain of AD patients but not of nondemented elderly control patients; Aβ activates the classical complement pathway in a standard complement activation assay and an ELISA complement activation assay, and Aβ activates the full classical pathway in vivo (Rogers et al., 1992, Proc. Natl. Acad. Sci. USA 89:10016-10020; Rogers et al., 1992, Res. Immunol. 143(6):624-630). Rogers et al. propose, based on several lines of direct and indirect evidence, that Aβ-mediated complement activation may be a pathogenic mechanism in AD (Rogers et al., 1992, Res. Immunol. 143(6):624-630).

Citation of a reference herein shall not be construed as an admission that such reference is prior art to the present invention.
3. SUMMARY OF THE INVENTION

The present invention relates to compounds that inhibit the binding of glycosaminoglycans and proteoglycans to beta amyloid peptide (A(beta); Aβ), and to compounds that inhibit Aβ activation of complement. In one aspect, the compound is a peptide having an amino acid sequence X-X-N-X, in which X is an amino acid with a cationic side chain and N is a neutral amino acid. In another aspect, the compound is a peptide having an amino acid sequence X-X-N-X-Z, in which X is an amino acid with a cationic side chain, and N and Z are each independently a neutral amino acid. In yet another aspect, the compound is peptide having an amino acid sequence X₁-N-X₂-X₃, in which at least two of X₁, X₂, and X₃ are independently an amino acid with an anionic side chain and the third X is an amino acid with an anionic side chain or a neutral amino acid, and N is independently a neutral amino acid. In yet a further aspect, the compound is an anionic disaccharide.

The present invention is based, in part, on the surprising discovery that relatively small molecules, such as peptides of 4 or 5 amino acids, and disaccharides, can inhibit the binding of Aβ with glycosaminoglycans and proteoglycans. Although not intending to be bound by any theory, the compounds of the invention are believed to reverse the glycosaminoglycan-mediated resistance of Aβ peptide to proteolysis, thus allowing natural degradation of the Aβ peptide and elimination of the Aβ plaques. Deposition of Aβ results in the formation of amyloid plaques in Alzheimer's disease.

The invention is also based, in part, on the surprising discovery that relatively small molecules, i.e., the compounds of the invention, inhibit
complement activation associated with beta-amyloid. Although not intending to be limited by any particular theory, it is believed that the molecules inhibit the interaction of C1q with Aβ.

The invention further relates to a pharmaceutical composition comprising one or more of the inhibitory compounds and a pharmaceutically acceptable carrier.

The invention also relates to a method for treating Alzheimer's disease comprising administering a therapeutically effective amount of a compound as described above to a subject suffering from Alzheimer's disease.

4. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. The amino acid sequence (SEQ ID NO:1) of the beta amyloid peptide (Aβ).

Figure 2. Heparin-induced aggregation of Aβ(1-28) examined as a function of pH. Aβ(1-28) at a concentration of 0.25 mM was incubated with 20 μM heparin in saline solutions at a variety of pH values. The percentage of soluble amyloid remaining in solution after incubation and centrifugation was determined for single samples at each pH value.

Figure 3. Heparin affinity chromatography of Aβ(1-28) at pH 4.0 (A) and 8.0 (B). Aβ(1-28) was injected onto a heparin affinity column as described in Section 6.1, infra, and non-bound peptide was eluted with either 20 mM sodium phosphate pH 8.0 or 20 mM sodium acetate pH 4.0. Bound peptide was eluted with a linear gradient of 0-0.5 M NaCl. Peptide elution was monitored by determining the absorbance at 280 nm.

Figure 4. The ability of heparin and heparan sulfate to cause aggregation of Aβ(1-28) as a function of glycosaminoglycan concentration. Aβ(1-28) at a
concentration of 0.5 mM was incubated with various concentrations of either heparin (open circles) or heparan sulfate (closed circles) at pH 4.0, and the amount of soluble peptide remaining after incubation and centrifugation was measured for single samples as described in Section 6.1, infra.

Figure 5. Comparison of the effects of various glycosaminoglycans on the solubility of Aβ(1-28) at pH 4.0 and 8.0. Aβ(1-28) at a concentration of 0.25 mM was incubated either in the absence (-GAG) or presence of 20 μM dermatan sulfate (light diagonal hatch), chondroitin sulfate (cross-hatch) or heparan sulfate (heavy diagonal hatch) at pH values of 4.0 and 8.0. Following centrifugation, the amount of peptide remaining soluble was determined at both pH's and expressed as a percentage of peptide found in non-centrifuged samples. All samples were assayed in triplicate, with the standard error of the mean shown for each condition.

Figure 6. Glycosaminoglycan-induced aggregation of Aβ(1-40) at pH 3.5 and 8.0. Aβ(1-40) at a concentration of 0.25 mM was incubated either in the absence (-GAG) (heavy diagonal hatch) or presence of heparan sulfate (HS) (cross hatch) or chondroitin sulfate (CS) (light diagonal hatch) at pH values of 3.5 and 8.0. After centrifugation, the amount of peptide remaining in solution was determined for each sample and expressed as a percentage of peptide found in non-centrifuged samples (solid bar). All samples were assayed in triplicate, with the standard error of the mean shown for each condition.

Figure 7. The binding of Aβ(13-17) to a heparin affinity column. Aβ(13-17) was injected onto a heparin affinity column as described in Section 6, infra, and non-bound peptide was eluted (solid line)
with either 20 mM sodium phosphate pH 4.0 (A) (open circles) or 20 mM sodium acetate pH 8.0 (B), (closed circles). Bound peptide was eluted with a linear gradient of 0-0.5 M NaCl (dotted line). Peptide elution was monitored by determining the absorbance at 230 nm of collected fractions.

Figure 8. Competition of Aβ(13-17) with Aβ(1-28) for heparin binding. A. Heparin concentration of 5 μM. B. Heparin concentration of 15 μM. Reaction solutions (20 μl, pH 4.0) contained 0.25 mM Aβ(1-28), 5.0 mM Aβ(13-17) and heparin, or 0.25 mM Aβ(1-28) either alone or with heparin as a control. The reactions were incubated 45 min prior to centrifugation. Each bar represents one of the duplicate assay samples. Samples 1 and 2 contained Aβ(1-28) alone; 3 and 4 contained Aβ(1-28) with heparin; and 5 and 6 contained Aβ(13-17), Aβ(1-28) and heparin.

Figure 9. Competition of Aβ(10-17) with Aβ(1-28) for heparin binding. All reactions (20 μl, pH 4.0) were incubated 45 min prior to centrifugation. Samples 1 and 2 contained Aβ(1-28). Sample 3 contained 0.25 mM Aβ(1-28) and 15 μM heparin. Samples 4 and 5 contained Aβ, heparin, and 5.0 mM Aβ(10-17).

Figure 10. Competition of Aβ(13-17) with Aβ(1-28) for chondroitin sulfate proteoglycan (CSPG) binding. Reaction solutions (10 μl, pH 4.0) were incubated 60 min. Samples 1 and 2 contained 0.25 mM Aβ(1-28); samples 3 and 4 contained 0.25 mM Aβ(1-28) and 8 μg CSPG; and samples 5 and 6 contained Aβ(1-28), CSPG, and 5.0 mM Aβ(13-17).

Figure 11. Competition of poly-L-lysine with Aβ(1-40) for heparin binding at pH 3.5. Samples 1 and 2 are Aβ(1-40); samples 3 and 4 are Aβ(1-40) with poly-L-lysine; samples 5 and 6 are Aβ(1-40) with
heparin and samples 7 and 8 are Aβ(1-40) with heparin and poly-L-lysine. Each bar represents a single sample. The assays were performed in duplicate. The specific concentrations of reagents and reaction conditions are described in the notes to Table 5, infra.

Figure 12. Competition of poly-L-lysine with Aβ(1-40) for CSPG binding at pH 3.5. Samples 1 and 2 are Aβ(1-40); samples 3 and 4 are Aβ(1-40) and CSPG; and samples 5 and 6 are Aβ(1-40), CSPG, and poly-L-lysine. Each bar represents a single sample. The assays were performed in duplicate. The specific concentrations of reagents and reaction conditions are described in the notes to Table 5, infra.

Figure 13. Effect of CSPG on the proteolysis of Aβ(1-40). Sample A contained 19.35 μg of Aβ(1-40); sample B contained Aβ(1-40) and papain (2 μg); sample C contained Aβ(1-40) and CSPG (30 μg), which were incubated together for 1 hour prior to the addition of papain. The amount of Aβ(1-40) was determined by densitometry after gel electrophoresis as described in Section 7.1.2, infra.

Figure 14. The effect of increasing concentration of CSPG on papain proteolysis of Aβ(1-40). Each sample contained 21.5 μg of Aβ(1-40) at pH 4.0. The amounts of CSPG added were 0, 24 μg, 48 μg, 72 μg and 96 μg, followed by a 1 hour incubation. The reaction mixtures were then incubated with 2 μg of papain for 12 hours, electrophoresed, and the amount of Aβ(1-40) quantified using a densitometer.

Figure 15. The effect of Aβ(13-16) and Aβ(13-17) on CSPG-mediated protection of Aβ(1-40) from papain proteolysis.

Figure 16. The effect of Aβ(20-24: Phe20 → Glu20) on CSPG-mediated protection of Aβ(1-40) from papain
proteolysis. Aβ(1-40) (4.3 µg) was treated with papain after a 45 minute incubation with CSPG (30 µg) and a subsequent incubation with the Aβ(20-24: Phe20 → Glu20) peptide Glu-Ala-Glu-Asp-Val (150 µg), all at pH 6.5. Control samples were incubated without the peptide, without the peptide or CSPG, and without the peptide, CSPG, and papain. Papain digestion ran for 18 hours, followed by gel electrophoresis. Aβ(1-40) bands were quantitated using a densitometer.

Figure 17. The effect of heparin disaccharide on CSPG-mediated protection of Aβ(1-40) from papain proteolysis. The reaction conditions were the same as described for Figure 17, with the exception that the heparin disaccharide (βUA-2s-[1→4]-GlcNS-6S) was used at 120 µg in place of the Aβ(20-24: Phe20 → Glu20) peptide.

Figure 18. A variation of a complement fixation assay, described in Section 8.1.1 infra, was utilized to assay for amyloid-induced complement activation. Complement activity is expressed as a percent of control samples which did not receive Aβ during the initial incubation. Two forms of peptides were used; fresh peptide (F1-40) was used within 3 days of solubilization and there was no evidence of appreciable aggregate formation, while aged peptide (A1-4) was solubilized and stored for 27 days before use. The aged peptide solutions were Congo Red birefringent, indicating that the peptide exists as fibrils.

Figure 19. A solid-phase binding assay for the evaluation of C1q binding to Aβ(1-28). This assay is described in Section 8.1.2, infra. The amount of C1q binding to the dotted Aβ(1-28) was detected by immunoenzyme staining and quantified through densitometric analysis of the stained membranes.
Figure 20. The effect of Aβ(13-16) on Aβ(1-40)-induced complement activation was investigated in the complement fixation assay described in Section 8.1.1, infra. Heat-aggregated human gamma globulin (AHGG) was included in the experiment since immunoglobulins are the normal activators of complement. Aβ(13-16) was added at 50-fold molar excess relative to Aβ(1-40).

Figure 21. The effect of Aβ(20-24: Phe20-Glu30) on Aβ(1-40)-induced complement activation was investigated using the complement fixation assay described in Section 8.1.1 infra. The pentapeptide was added at 12.5 to 50-fold molar excess relative to Aβ(1-40).

Figure 22. Clq binding to Aβ(25-35) and Aβ(1-40) was investigated using the methodology described in Section 8.1.2, infra.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a compounds that inhibit the binding of glycosaminoglycans and/or proteoglycans to beta amyloid peptide (A(β); SEQ ID NO:1), and to compounds that inhibit Aβ activation of complement, and therapeutic methods and compositions based thereon.

5.1. INHIBITORY COMPOUNDS OF THE INVENTION

The inhibitory compounds of the invention are relatively small molecules, such as tetra- to hexapeptides or sulfated disaccharides that have the ability to inhibit the aggregation of Aβ with proteoglycans or glycosaminoglycans. Preferably, the molecules of the invention consist of a sequence of not greater than 6 amino acid residues, and generally not greater than 8 amino acid residues, and, in a
particular embodiment, comprise the sequences of peptides described in the subsections below. In a preferred aspect, hydrophobic residues are added to the peptides of the invention, to enhance the ability of the peptides to cross the blood-brain barrier.

Alternatively, the compounds of the invention have the ability to inhibit Aβ-mediated activation of complement.

Specific tests for the ability to inhibit the aggregation of Aβ with glycosaminoglycans; to reverse the glycosaminoglycan-mediated protection of Aβ from proteolysis; and to inhibit Aβ activation of complement are described in the Examples in Sections 6, 7 and 8, respectively. Accordingly, the compounds of the invention can inhibit or reverse the neuropathology associated with Alzheimer's disease. Although not intending to be limited to any particular theory, it is believed that the neuropathology can result from beta amyloid deposition, the inhibitory effects of proteoglycans and glycosaminoglycans in amyloid on nerve growth, and/or inappropriate complement activation that can destroy nerve cells. The present invention provides three classes of compounds that can inhibit aggregation of Aβ and glycosaminoglycans, or inhibit Aβ-mediated complement activation, or both, described in detail below.

The peptide inhibitory compounds of the invention are preferably prepared using standard synthetic chemistry from, preferably, naturally occurring amino acids, or obtained commercially.

However, the peptides of the invention can also contain non-natural amino acids or cyclic peptides. Non-classical amino acids include but are not limited to the D-isomers of the common amino acids, α-amino isobutyric acid, 4-aminobutyric acid, hydroxyproline,
sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, designer amino acids such as β-methyl amino acids, Ca-methyl amino acids, Na-methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be the D (dextrorotary) or L (levorotary) amino acid.

The peptide may be prepared by methods that are known in the art. For example, in brief, solid phase peptide synthesis consists of coupling the carboxyl group of the C-terminal amino acid to a resin and successively adding N-alpha protected amino acids. The protecting groups may be any known in the art. Before each new amino acid is added to the growing chain, the protecting group of the previous amino acid added to the chain is removed. The coupling of amino acids to appropriate resins is described by Rivier et al., U.S. Patent No. 4,244,946. Such solid phase syntheses have been described, for example, by Merrifield, 1964, J. Am. Chem. Soc. 85:2149; Vale et al., 1981, Science 213:1394-1397; Marki et al., 1981, J. Am. Chem. Soc. 103:3178 and in U.S. Patent Nos. 4,305,872 and 4,316,891. In a preferred aspect, an automated peptide synthesizer is employed.

Purification of the synthesized peptides can be carried out by standard methods including chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. In a preferred embodiment, reverse phase HPLC (high performance liquid chromatography) is employed.

The disaccharide compounds can be prepared synthetically by standard procedures or obtained commercially.
5.1.1 THE HEPARIN BINDING DOMAIN OF Aβ

In one embodiment, the inhibitory compound is a peptide that competes with Aβ for binding to a glycosaminoglycan or proteoglycan, and therefore inhibits Aβ binding thereto. Although not intending to be bound by any particular theory, it is believed that aggregation of Aβ with proteoglycans and glycosaminoglycans results in protection of the Aβ from proteases, and thus prevents the clearance of such amyloid deposits, resulting in neuropathology. It is also believed that the glycosaminoglycans or proteoglycans can directly contribute to neuropathology by inhibiting nerve growth and regeneration.

In a specific embodiment, infra, the inhibitory compound of the invention is a peptide of four or five amino acids based on the putative heparin binding domain of Aβ. The sequence of the heparin binding domain of Aβ is histidine₁₅-histidine₁₆-glutamine₁₅-lysine₁₆-leucine₁₇. In the examples sections infra, peptides corresponding to Aβ(13-17) and Aβ(13-16) were able to inhibit the binding of Aβ to heparin, chondroitin sulfate proteoglycan, and dextran sulfate, as shown in aggregation assays and by the ability of the peptides to reverse proteoglycan/glycosaminoglycan-mediated protection of Aβ from papain proteolysis.

In one aspect, the compound is a peptide having an amino acid sequence X-X-N-X, in which X is a amino acid with a cationic side chain and N is a neutral amino acid. Preferably, X is selected from the group consisting of histidine, lysine and arginine, and N is selected from the group consisting of glycine, alanine, valine, serine, threonine, asparagine, glutamine, methionine, and cysteine, and hydrophobic
amino acids such as isoleucine, phenylalanine, and leucine. In a preferred embodiment, the peptide has the amino acid sequence histidine-histidine-glutamine-lysine, which corresponds to Aβ(13-16). In another embodiment, the peptide has the sequence X-X-X-X, with X as defined above; in a specific embodiment, such a peptide has the sequence Lys-Lys-Lys-Lys (SEQ ID NO:2) or His-His-His-His (SEQ ID NO:3).

In another aspect, the compound is a peptide having an amino acid sequence X-X-N-X-Z, in which X is a amino acid with a cationic side chain, N and Z are each independently a neutral amino acid. Preferably, X is selected from the group consisting of histidine, lysine and arginine, N is selected from the group consisting of glycine, alanine, valine, serine, threonine, asparagine, glutamine, methionine, cysteine, and hydrophobic amino acids such as isoleucine, phenylalanine, and leucine, and Z is selected from the group consisting of glycine, alanine, valine, leucine, isoleucine, serine, threonine, asparagine, glutamine, tryptophane, tyrosine, phenylalanine, methionine and cysteine. In a preferred embodiment, the peptide has the amino acid sequence histidine-histidine-glutamine-lysine-leucine, which corresponds to Aβ(13-17).

Any basic amino acid, including but not limited to histidine, lysine, asparagine, di-aminobutaric acid, and amino acid analogs having amines, guanidines, or other basic side chains and D-amino acids, can be substituted for any of the basic amino acids corresponding to positions 13, 14 and 16 of the Aβ heparin binding domain.

Any neutral (i.e., uncharged) amino acid can be substituted for the amino acid (N) corresponding to the 15 position of the Aβ peptide. For example, the
amino acids glycine, alanine, valine, serine, threonine, asparagine, glutamine, methionine, cysteine, isoleucine, phenylalanine, and leucine, as well as analogs and optical isomers thereof, can be used at that position.

5.1.2 THE 20-24 REGION OF Aβ

In another embodiment, the compound of the invention is a peptide that competes with glycosaminoglycans for binding to the heparin binding domain of Aβ. In particular, although not intending to be bound by any particular theory, it is believed that the 20-24 region of Aβ binds to the heparin binding domain of Aβ in an antiparallel fashion. A pentapeptide having the sequence Phe-Ala-Glu-Asp-Val or an analog thereof and thus corresponding to the amino acids 20-24 region of Aβ is specifically preferred since such a peptide will target Aβ, whereas a peptide corresponding to Aβ(13-16) or (13-17) (see Section 5.1.1) will target all glycosaminoglycans or proteoglycans.

In specific example, infra, the peptide corresponding to the 20-24 region of Aβ and having the structure phenylalanine-alanine-glutamic acid-aspartic acid reversed the glycosaminoglycan-mediated protection of Aβ from proteolysis. In another specific example, an analog of the 20-24 region, in which phenylalanine was substituted with glutamic acid (SEQ ID NO:6) or aspartic acid (SEQ ID NO:7) had the same activity.

In one aspect, the compound is a peptide having an amino acid sequence X₁-N-X₂-X₃, in which at least two of X₁, X₂, and X₃ are independently an amino acid with an anionic side chain, and the third X is an amino acid with an anionic side chain or a neutral amino
acid, and N is independently a neutral amino acid. Preferably, the amino acid with the anionic side chain is selected from the group consisting of aspartic acid and glutamic acid or other anionic amino acid analog known to one skilled in the art, and the non-anionic X and N are independently selected from the group consisting of any neutral amino acid, but preferably glycine, alanine, valine, serine, threonine, asparagine, glutamine, methionine, cysteine, and the hydrophobic amino acids isoleucine, phenylalanine, and leucine. In a specific embodiment, the peptide has the amino acid sequence phenylalanine-alanine-glutamic acid-aspartic acid, which corresponds to Aβ(20-23). In another specific embodiment, the peptide has the amino acid sequence aspartic acid-alanine-glutamic acid-aspartic acid (SEQ ID NO:4). In yet another embodiment, the peptide has the amino acid sequence glutamic acid-alanine-glutamic acid-aspartic acid (SEQ ID NO:5).

A pentapeptide is also provided, having the sequence X₁-N-X₂-X₃-B, with X₁, N, X₂, and X₃ as defined above, and B being any hydrophobic amino acid, including but not limited to leucine, valine, isoleucine, and phenylalanine. In this peptide corresponding to the 20-24 region of Aβ, only two acidic amino acids (with an anionic side chain) need to be found in the pentapeptide; preferably, however, there are three acidic amino acids, to act as complements to the three basic amino acids in the heparin binding domain of Aβ.

The non-anionic amino acid in the structure can be any neutral amino acid, but preferably glycine, alanine, valine, serine, threonine, asparagine, glutamine, methionine, cysteine, isoleucine, phenylalanine, and leucine.
5.1.3 THE 25-35 REGION OF Aβ

In another embodiment, the compound of the invention is a peptide that inhibits Aβ-induced activation of the complement cascade. In a specific embodiment, such a peptide is Aβ(13-17) or an analog thereof. In another specific embodiment, such a peptide is Aβ(25-35) or an analog thereof.

5.1.4 SULFATED DISACCHARIDES

In another embodiment, the compound of the invention is a sulfated disaccharide that competes with glycosaminoglycans for binding to the heparin binding domain of Aβ. In particular, although not intending to be bound by any particular theory, it is believed that the sulfated disaccharide binds to the heparin binding domain of Aβ. Such a disaccharide is preferred since it will target Aβ specifically instead of glycosaminoglycans and proteoglycans generally.

Thus, in yet a further aspect, the compound is an anionic disaccharide. Although any sulfated disaccharide is envisioned, in a specific embodiment the disaccharide is derived from heparin. In one embodiment, sulfated disaccharides of the invention contain either uronic or glucuronic acid. More particularly, the disaccharide is α-4-deoxy-L-threo-hex-4-enopyranosyluronic acid-[1→4] D-glucosamine-N-sulfate-6-sulfate.

5.2. MODIFICATION OF THE COMPOUNDS OF THE INVENTION

In a preferred embodiment, the compounds of the invention are modified so as to permit or enhance their ability to cross the blood brain barrier. Such compounds would be preferred for oral or parenteral administration other than intraventricularly. Suitable modifications of the compounds to enhance
their ability to cross the blood-brain barrier include, but are not limited to, adding hydrophobic amino acids, coupling the compound to a lipid, coupling to transferrin, coupling to an antibody which recognizes the transferrin receptor, coupling to avidin, etc. Preferably, the chemical linkage effectuating the coupling is labile, e.g., a disulfide bond.

Other modifications of the peptides of the invention, such as chemical modifications known in the art can be carried out, e.g., acetylation, amidation, phosphorylation, etc. In a specific embodiment, acetylation of the amino terminus and/or amidation of the carboxy-terminus are carried out.

5.3. DEMONSTRATION OF THERAPEUTIC UTILITY

The peptides or disaccharides are tested in vitro and then preferably in vivo for the desired therapeutic utility.

Any in vitro assay known in the art can be used to detect inhibition of proteoglycan/glycosaminoglycan bind to Aβ, or inhibition of complement activation by a peptide disaccharide of the invention. In a preferred aspect, the assays described in the examples sections infra are employed.

Peptides or disaccharides demonstrated to have the desired activity in vitro can be tested in vivo for the desired inhibitory activity. For example, such compounds can be tested in suitable animal model systems prior to testing in humans, including but not limited to rats, mice, chicken, cows, monkeys, rabbits, etc. Suitable model systems are also used to demonstrate therapeutic utility.
For in vivo testing, prior to administration to humans, any animal model system known in the art may be used.

5.4. PHARMACEUTICAL COMPOSITIONS

According to the present invention, the peptide or disaccharide inhibitory compounds of the invention can be preferably prepared as a pharmaceutical composition with a pharmaceutically acceptable carrier for administration to a subject. As used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, magnesium carbonate, magnesium stearate, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. These compositions can take the form of solutions, suspensions, tablets, pills, capsules, powders, sustained-release formulations and the like. Suitable
pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain a therapeutically effective amount of the active compound, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient.

In a specific embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The peptides or disaccharides of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium,
ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

5.5. THERAPEUTIC ADMINISTRATION OF THE COMPOUNDS

The compounds of the present invention can be used for the treatment of symptoms of amyloidosis associated with Alzheimer's disease or other diseases including but not limited to AA (inflammation-associated)-amyloid, AL-amyloid (amyloid with deposition of immunoglobulin light chains), Down's syndrome, and prion diseases such as Creutzfeldt-Jakob disease, Gerstmann-Straussler syndrome, kuru, and scrapie. Preferably, the compounds of the invention are used for treatment of Alzheimer's disease (AD). It is contemplated that peptide or disaccharide compounds can be administered to a subject in need of prophylactic or therapeutic treatment. As used herein, the term "subject" refers to an animal, more preferably a mammal, and most preferably a human. Thus, the therapeutic treatment can commence with diagnosis of AD, or the onset of AD, according to the appropriate criteria. Once a subject has been identified as having a need for therapeutic treatment
of AD, a therapeutically effective dose of a compound of the invention can be administered to the subject.

What constitutes a therapeutically effective amount in a particular case will depend on a variety of factors within the knowledge of the skilled practitioner. Such factors include the physical condition of the subject being treated, the severity of the condition being treated, the disorder or disease being treated, and so forth.

The peptide or disaccharide compounds, particularly those optimized to cross the blood-brain barrier, can be administered systemically, and more preferably parenterally, i.e., via an intraperitoneal, intravenous, perioral, subcutaneous, intramuscular, intraarterial, etc. route, in order to treat Alzheimer's disease. Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The peptides or disaccharides may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. In a preferred aspect, the compounds are directly administered to the cerebrospinal fluid by intraventricular injection.

Pulmonary administration can also be employed.

In a specific embodiment, it may be desirable to administer the peptides or disaccharides of the invention locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, by injection, by means of a catheter, by
means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers.

In another embodiment, the therapeutic compound can be delivered in a vesicle, in particular a liposome (see Langer, Science 249:1527-1533 (1990); Treat et al., in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); Lopez-Berestein, ibid., pp. 317-327; see generally ibid.)

In yet another embodiment, the therapeutic compound can be delivered in a controlled release system. In one embodiment, a pump may be used (see Langer, supra; Sefton, CRC Crit. Ref. Biomed. Eng. 14:201 (1987); Buchwald et al., Surgery 88:507 (1980); Saudek et al., N. Engl. J. Med. 321:574 (1989)). In another embodiment, polymeric materials can be used (see Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, J. Macromol. Sci. Rev. Macromol. Chem. 23:61 (1983); see also Levy et al., Science 228:190 (1985); During et al., Ann. Neurol. 25:351 (1989); Howard et al., J. Neurosurg. 71:105 (1989)). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, i.e., the brain, thus requiring only a fraction of the systemic dose (see, e.g., Goodson, in Medical Applications of Controlled Release, supra, vol. 2, pp. 115-138 (1984)).

Other controlled release systems are discussed in the review by Langer (Science 249:1527-1533 (1990)).
The invention can be better understood by reference to the following examples, which are provided merely by way of exemplification and are not intended to limit the invention.

6. EXAMPLE: pH-DEPENDENT BINDING OF SYNTHETIC β-AMYLOYD PEPTIDES TO GLYCOSAMINOGLYCANS

In this example, we have tested whether a Aβ(12-17) peptide, having the sequence Val-His-His-Gln-Lys-Leu, interacts with glycosaminoglycans in a pH-dependent manner; in particular, whether there is increased association of the peptide with the sulfated polysaccharides below pH 6-7 where the histidine residues at position 13 and 14 would be protonated. This example also reports on the ability of peptides to compete with Aβ peptide or on Aβ(1-28) peptide for binding to heparin and chondroitin sulfate proteoglycan.

6.1. MATERIALS AND METHODS
6.1.1 Aβ AGGREGATION ASSAY

The association of Aβ peptides with glycosaminoglycans was evaluated by analyzing the amount of peptide that would remain soluble after incubation with the polysaccharides followed by centrifugation. Amyloid peptides (stored as 2 mM stock solutions in water) were diluted to concentrations of 0.25 or 0.5 mM and mixed with glycosaminoglycans (concentrations ranging from 0.1-100 µM) for approximately 1 h at room temperature (21-24°C) in either 20 mM sodium acetate pH 3.5, 4.0 or 5.0 with 150 mM NaCl, or 20 mM sodium phosphate pH 6.0, 7.0 or 8.0 containing 150 mM NaCl. The precise concentrations of amyloid and glycosaminoglycans used for individual experiments are noted in the figure legends and Tables. Typically, the final volume of
the mixtures was 10 or 20 μl. The peptides used were Aβ(1-28) ([glyln]β-amyloid; Bachem, Inc.), Aβ(1-40) (synthesized by Biosynthesis, Inc.), Aβ(25-35) (Bachem, Inc.), and Aβ(13-17) (synthesized as described infra). The glycosaminoglycans employed were heparin (porcine intestinal mucosa; CalBiochem, Inc.), heparan sulfate (bovine kidney; Seikagaku Corp.), chondroitin sulfate (bovine trachea chondroitin sulfate A; Sigma Chemical Co.), and dermatan sulfate (bovine mucosa chondroitin sulfate B; Sigma Chemical Co.). In addition to the naturally occurring glycosaminoglycans, the polymers dextran sulfate (mol. wt. = 5,000; Sigma Chemical Co.) and dextran (mol. wt. = 10,200; Sigma Chemical Co.) were used in the aggregation assay. Poly-L-lysine, molecular weight 30-70,000, was obtained from ICN. Subsequent to the incubation period, the mixtures were centrifuged in a microfuge at 10,000 x g for 10 min, and aliquots of the resulting supernatants were assayed for absorbance at 230 nm. As controls, samples were prepared without glycosaminoglycan addition and treated as above to determine if amyloid peptides were sedimenting in the absence of polysaccharide. The absorbance values obtained for the controls and samples were compared to the absorbance of peptide in the appropriate buffer that had not undergone centrifugation. The values obtained were expressed as percent soluble amyloid.

6.1.2 HEPARIN AFFINITY CHROMATOGRAPHY

Aβ(1-28), Aβ(25-35) and Aβ(13-17) were injected onto a heparin affinity column (Bio-Rad Econo-Pac cartridge; 5 ml volume) that was equilibrated with either 20 mM sodium acetate, pH 4.0 or 20 mM sodium phosphate, pH 8.0. The peptides were at a
concentration of 0.25 mM in water, with 0.2 ml injected. Non-bound peptides were eluted from the column with approximately 15 ml of the equilibration buffer (flow rate of 1 ml/min), and bound peptides were then eluted with a linear gradient of 0 to 0.5 M NaCl in equilibration buffer over 25 min. The change in NaCl concentration was monitored with an in-line conductivity meter. The elution of Aβ(1-28) was monitored at 280 nm. For Aβ(25-35), and Aβ(13-17), 2 ml fractions were collected from the column and assayed for absorbance at 230 nm using a spectrophotometer.

6.1.3 COMPETITION ASSAYS

Aggregation assays with Aβ(1-28) or Aβ(1-40) were performed as described in Section 6.1.1, supra, in the presence of excess amounts of test peptides. The test peptides for inhibition of aggregation were Aβ(13-17), Aβ(10-17), Aβ(1-28) (tested for its ability to inhibit aggregation of Aβ(1-40), and poly-L-lysine, typically at 5.0 mM (about a 20-fold molar excess) concentration.

Reactions were incubated for 45 minutes to three hours at room temperature, then centrifuged for 10 minutes at 14,000 rpm in a microfuge. Five to 10 μl of each supernatant was carefully removed and added to an equal volume of Tris-Tricine sample buffer (8% SDS, 24% glycerol, 0.1 M Tris base, 0.1 M Tricine, 0.05% Bromophenol Blue). Samples were analyzed on 16.5% Tris-Tricine gel (16.5% acrylamide, 1 M Tris base, 0.1% SDS, 13.3% glycerol, pH 8.45) with a 4% stacking gel (4% acrylamide, 1 M Tris base, 0.1% SDS, pH 8.45). The anode buffer used was 0.2 M Tris base, pH 8.9, and the cathode buffer was 0.1 M Tris base, 0.1 M Tricine, 0.1% SDS. The gels were stained with 0.2% Coomassie
brilliant blue in 45% methanol and 10% acetic acid, then destained in 45% methanol and 10% acetic acid. The gels were scanned with densitometer and Aβ(1-28) or Aβ(1-40) protein bands were quantified.

6.1.4 SYNTHESIS OF Aβ(13-17)

Aβ(13-17) was synthesized by stepwise elongation from the COOH-terminus using solid-phase methodology (Merrifield, 1963, J. Am. Chem. Soc. 85:2149-2154). Rink resin (Rink, 1987, Tetrahedron Lett. 28:3787-3790) was used as the solid support and fluorenlymethoxycarbonyl (Carpino and Han, 1970, J. Am. Chem. Soc. 92:5748-5749; Carpino and Han, 1972, J. Org. Chem. 37:3404-3409) moieties for protection of the amino groups. The side chains of lysines were protected with butoxycarbonyl and those of histidines with fluorenlymethoxycarbonyl. All amino acid residues except glutamine were introduced using diisopropylcarbodiimide hydroxybenzotriazole; for glutamine, pentafluorophenyl ester was employed. Fluorenlymethoxycarbonyl groups were removed with piperidine-dimethylformamide and the desired peptide sequences were cleaved from the solid support with trifluoroacetic acid. The resulting peptides were purified by Sephadex G10 chromatography followed by C18-reversed phase HPLC.

6.2. RESULTS

6.2.1 pH-DEPENDENT INTERACTION OF HEPARIN WITH Aβ

To assay for the association of Aβ(1-28) with heparin, we mixed aliquots of amyloid and heparin solutions together in aqueous saline solutions buffered at pH values ranging from 4 through 8. Visual inspection of the mixtures revealed that the more acidic solutions contained a precipitate which could
be sedimented by centrifugation. Spectrophotometric analysis of the supernatant at 230 nm following centrifugation gave a measure of the amount of remaining soluble peptide, and a plot of this value as a function of pH is shown in Fig. 2. The ability of the heparin to interact with Aβ(1-28) and cause precipitation was pH-dependent, with increased aggregation below pH 7. Essentially no precipitable material was seen at pH 8. The pH-dependent precipitation of Aβ(1-28) was due to heparin and not to a gross change in the solubility of the peptide itself, since we did not observe sedimentation of Aβ(1-28) in the absence of glycosaminoglycan at any of the pH values tested. These observations are consistent with the involvement of one or more histidine residues in heparin binding, and suggest that the histidines at residues 13 and/or 14 of Aβ(1-28) are involved in the interaction with the glycosaminoglycan.

To confirm that the aggregation of Aβ(1-28) by heparin was a reflection of pH-dependent binding of the glycosaminoglycan to the peptide, the association of Aβ(1-28) with a heparin affinity column was investigated as a function of pH. As noted in Fig. 3, Aβ(1-28) bound to the heparin matrix at pH 4.0 and was eluted with a NaCl gradient (elution at approximately 0.22 M NaCl). In contrast, the vast majority of the peptide was not retained on the affinity column at pH 8.0, and that peptide which did bind was eluted with a low concentration of NaCl (0.04 M). These data support the notion that Aβ interacts with heparin in a pH-dependent fashion.
6.2.2 GLYCOSAMINOGLYCAN SPECIFICITY
OF THE Aβ INTERACTION

The pH-dependent interaction of heparin with Aβ(1-28) implied that this highly sulfated glycosaminoglycan was associating with the consensus heparin-binding domain located at residues 12-17 of the peptide. The binding of heparin to Aβ(1-28), with resulting aggregation, was concentration-dependent (Fig. 4). With a peptide concentration of 0.5 mM at pH 4.0, appreciable precipitation occurred over a range of 2-50 μM heparin. Although heparin resembles the heparan sulfate glycosaminoglycan chains of HSPG in that both contain predominantly N-acetylgalactosamine and uronic acids as constituent monosaccharides, the former has a higher degree of sulfation than the HSPG polysaccharides. To determine whether the observed interaction of heparin with Aβ was a result of this increased level of sulfation, we examined the more physiologically relevant polysaccharide, heparan sulfate, in the aggregation assay at pH 4.0. As demonstrated in Fig. 4, heparan sulfate and heparin showed nearly identical dose-dependent aggregations of Aβ(1-28).

While heparan sulfate appeared to be comparable to heparin in its ability to precipitate Aβ(1-28), it was of interest to determine whether glycosaminoglycans containing differing monosaccharide units could also interact with the amyloid peptide. Chondroitin sulfate and dermatan sulfate both contain N-acetylgalactosamine residues in addition to uronic acids in their polysaccharide chains. These two glycosaminoglycans caused a level of aggregation of Aβ(1-28) at pH 4.0 that was similar to that seen with heparan sulfate (Fig. 5). None of the sulfated polysaccharides caused significant precipitation of
Aβ(1-28) at pH 8.0, consistent with the involvement of one or more histidine residues in the interaction.

The ability of a variety of glycosaminoglycans to bind to Aβ(1-28) at pH values below neutrality indicated that the association was relatively sugar non-specific, and implied that the binding was mediated through ionic interaction of sulfate groups with the positively charged amino acids of the peptide. To further address the nature of the glycosaminoglycan-Aβ interaction, we examined whether the polymer, dextran sulfate, could also bind to and aggregate Aβ(1-28). As with the sulfated glycosaminoglycans, dextran sulfate addition to Aβ(1-28) at pH 4.0 resulted in appreciable precipitation of the peptide (Table 1). Further evidence that this binding was due to the sulfate groups of the polysaccharide is seen in the inability of dextran to cause aggregation of Aβ (Table 1).

<table>
<thead>
<tr>
<th>Table 1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Comparison of Dextran Sulfate- and Dextran-Induced Aggregation of Aβ(1-28)</strong></td>
</tr>
<tr>
<td><strong>Polymer Added</strong></td>
</tr>
<tr>
<td>Dextran Sulfate</td>
</tr>
<tr>
<td>Dextran</td>
</tr>
</tbody>
</table>

Aβ(1-28) at a concentration of 0.25 mM was incubated with either 0.4 mg/ml dextran sulfate (80 μM) or 0.4 mg/ml dextran (40 μM) at pH 4.0. The percentage of peptide remaining in solution following centrifugation was then determined as described in Materials and Methods.
6.2.3 ASSOCIATION OF GLYCOSAMINOGLYCANS WITH OTHER Aβ PEPTIDES

The association of Aβ(1-28) with glycosaminoglycans suggested that such an interaction may be relevant to the localization of proteoglycans within the senile plaques of AD brain. The amyloid peptide of the AD plaques is 40-43 amino acids in length (SEQ ID NO:1) (Masters et al., 1985, EMBO J. 4:2757-2763; Mori et al., 1992, J. Biol. Chem. 266:12878-12883). We examined whether Aβ(1-40) could be precipitated by glycosaminoglycans. Addition of either heparan sulfate or chondroitin sulfate to Aβ(1-40) at pH 8.0 resulted in essentially no aggregation and precipitation of the peptide (Fig. 6). When Aβ(1-40) was incubated at pH 4.0 in the absence of glycosaminoglycan, we noted that approximately 55% of the peptide could be sedimented. The amount of Aβ(1-40) that was precipitable in the absence of glycosaminoglycan increased to more than 85% at pH values of 5.0 and 6.0 (data not shown). This increased insolubility of the peptide between pH 4-7 is consistent with previous reports (Barrow and Zagorski, 1991, Science 253:179-182; Zagorski and Barrow, 1992, Biochemistry 31:5621-5631), and thus precluded accurate determination of glycosaminoglycan-induced aggregation of the peptide at pH 4-7. At pH 3.5, Aβ(1-40) remained soluble after centrifugation in the absence of glycosaminoglycan (Fig. 6). Addition of heparan sulfate or chondroitin sulfate to Aβ(1-40) at pH 3.5 caused a significant increase in the amount of peptide that could be sedimented (Fig. 6), indicating that the sulfated polysaccharides were interacting with the amyloid peptide.

To investigate whether the glycosaminoglycan-binding domain of Aβ resides within residues 12-17, a
pentapeptide containing the His-His-Gln-Lys-Leu sequence corresponding to amino acids 13-17 was analyzed for its ability to bind to a heparin affinity column at pH 4.0 and 8.0. As with Aβ(1-28), this short peptide showed an acidic pH requirement for heparin association (Fig. 7), with little binding to the column at pH 8.0. Essentially all of the Aβ(13-17) bound to the heparin column at pH 4.0, and the peptide was eluted at a NaCl concentration of approximately 0.45 M. This binding was tighter than that seen with Aβ(1-28), presumably reflecting a greater accessibility of the charged residues of the pentapeptide to the heparin matrix. In contrast to the results seen with Aβ(13-17), a peptide containing residues 25-35 of the amyloid peptide sequence did not bind to a heparin affinity column at pH 4.0 (data not shown). This is consistent with the localization of the glycosaminoglycan-binding domain within the first 25 amino acids of the amyloid peptide.

6.2.4 COMPETITION OF Aβ PEPTIDES FOR BINDING GLYCOSAMINOGLYCANS

Peptides corresponding to the heparin binding domain of Aβ were tested for the ability to inhibit binding of Aβ(1-28) or Aβ(1-40) to the glycosaminoglycans heparin and chondroitin sulfate proteoglycan. The aggregation assay was used to demonstrate binding of the Aβ peptide to the glycosaminoglycan.

Aβ(13-17) inhibited binding of Aβ(1-28) to heparin at both 5 and 15 μM (Fig. 8). The amount of free Aβ(1-28) decreased by precipitating with heparin, and increased in the presence of Aβ(13-17), which indicates that Aβ(13-17) inhibited the binding of Aβ(1-28) with heparin. The results shown in Fig. 8 are summarized below in Table 2.
### Table 2

**Competition of Aβ(13-17) With Aβ(1-28) For Heparin Binding**

<table>
<thead>
<tr>
<th>Sample - 5μM Heparin</th>
<th>Avg. O.D.</th>
<th>% Soluble</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-28</td>
<td>0.645</td>
<td>100</td>
</tr>
<tr>
<td>1-28 + Heparin</td>
<td>0.336</td>
<td>52</td>
</tr>
<tr>
<td>1-28 + Heparin + 13-17</td>
<td>0.503</td>
<td>78</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample - 15μM Heparin</th>
<th>Avg. O.D.</th>
<th>% Soluble</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-28</td>
<td>0.807</td>
<td>100</td>
</tr>
<tr>
<td>1-28 + Heparin</td>
<td>0.086</td>
<td>11</td>
</tr>
<tr>
<td>1-28 + Heparin + 13-17</td>
<td>0.395</td>
<td>49</td>
</tr>
</tbody>
</table>

Inhibition of Aβ(1-28) binding to heparin was also tested with an Aβ(10-17) peptide (Fig. 9 and Table 3). The results of this assay indicate that Aβ(10-17) does not compete with Aβ(1-28) for heparin binding at pH 4, although Aβ(13-17) clearly does. It is possible that differences in the charge density of the Aβ(10-17) octapeptide are responsible for its inability to compete with Aβ(1-28) for heparin binding.
Table 3

<table>
<thead>
<tr>
<th>Sample</th>
<th>Avg. O.D.</th>
<th>% Soluble</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-28</td>
<td>4.028</td>
<td>100</td>
</tr>
<tr>
<td>1-28 + Heparin</td>
<td>0.099</td>
<td>2</td>
</tr>
<tr>
<td>1-28 + Heparin + 10-17</td>
<td>0.087</td>
<td>2</td>
</tr>
</tbody>
</table>

Competition of Aβ(13-17) with Aβ(1-28) for binding the proteoglycan chondroitin sulfate (CSPG) was tested at pH 4.0 (Fig. 10; Table 4). Aβ(1-28) aggregated with CSPG. The aggregation was inhibited by Aβ(13-17), thus indicating that Aβ(13-17) competes with Aβ(1-28) for CSPG binding.

Table 4

<table>
<thead>
<tr>
<th>Sample</th>
<th>Avg. O.D.</th>
<th>% Soluble</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-28</td>
<td>2.585</td>
<td>100</td>
</tr>
<tr>
<td>1-28 + CSPG</td>
<td>0.050</td>
<td>2</td>
</tr>
<tr>
<td>1-28 + CSPG + 13-17</td>
<td>0.725</td>
<td>28</td>
</tr>
</tbody>
</table>

To investigate whether the competition for binding to the glycosaminoglycans was dependent solely on ionic interactions, poly-L-lysine (MW 30,000-70,000) was tested for the ability to compete with Aβ(1-40) for binding to heparin and to CSPG. Poly-L-lysine, which had no effect upon or slightly enhanced Aβ(1-40) solubility, did compete with Aβ(1-40) for
binding heparin (Fig. 11) and CSPG (Fig. 12). These results are summarized in Table 5.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Competitor</th>
<th>Avg. O.D.</th>
<th>% Soluble</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 1-40 only</td>
<td>--</td>
<td>0.906&lt;sup&gt;2&lt;/sup&gt;</td>
<td>100</td>
</tr>
<tr>
<td>2. 1-40 only</td>
<td></td>
<td>1.055&lt;sup&gt;3&lt;/sup&gt;</td>
<td>100</td>
</tr>
<tr>
<td>3. 1-40 + Poly-L-Lys&lt;sup&gt;4&lt;/sup&gt;</td>
<td>--</td>
<td>1.015</td>
<td>112</td>
</tr>
<tr>
<td>4. 1-40</td>
<td>Heparin&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.004</td>
<td>0</td>
</tr>
<tr>
<td>5. 1-40 + Poly-L-Lys</td>
<td>Heparin</td>
<td>0.424</td>
<td>47</td>
</tr>
<tr>
<td>6. 1-40</td>
<td>CSPG&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0.021</td>
<td>2</td>
</tr>
<tr>
<td>7. 1-40 + Poly-L-Lys</td>
<td>CSPG</td>
<td>0.430</td>
<td>41</td>
</tr>
</tbody>
</table>

1. Samples (10 μl, pH 3.5) were incubated 45 min. The fresh Aβ(1-40) was used.
2. This value was obtained from the control of the heparin binding assays and was used to normalize to 100% for samples 3-5.
3. This value was obtained from the control of the CSPG binding assays and was used to normalize to 100% for samples 6 and 7.
4. Poly-L-lysine was present at 6 μg per sample.
5. The concentration of heparin was 15 μM.
6. CSPG was present at 4 μg per sample.

6.3. DISCUSSION

We found that a variety of glycosaminoglycan chains bind to and aggregate Aβ(1-28). The interaction of these sulfated polysaccharides with Aβ(1-28) increases as the pH value is lowered below 6-
7, and there is essentially no association of the peptide with the glycosaminoglycans at pH 8. These data are consistent with the involvement of one or more positively charged histidine residues in the binding of the peptide to the sulfated sugar moieties, and suggest that the interaction is primarily ionic in nature. This latter interpretation is supported by the observation that the anionic polymer, dextran sulfate, can bind the amyloid peptide whereas the non-charged polysaccharide, dextran, cannot.

In addition to the ability of $\alpha\beta(1-28)$ to bind various sulfated polysaccharides, we have shown that $\alpha\beta(1-40)$ also associates with glycosaminoglycans. In contrast, a peptide containing residues 25-35 of the amyloid peptide does not show appreciable association with heparin. We propose that the glycosaminoglycan binding site is found at residues 12-17 of the amyloid peptide (also see Fraser et al., 1992, J. Neurochem. 59:1531-1540; and Kisilevsky, 1989, Neurobiol. Aging 10:499-500). This region of the peptide contains the consensus heparin binding sequence (Cardin and Weintraub, 1989, Arteriosclerosis 9:21-32) Val-His-His-Gln-Lys-Leu, and the ionization state of one or both of the tandem histidines could modulate glycosaminoglycan binding. Supporting this conclusion is our observation that a pentapeptide consisting of the $\alpha\beta(13-17)$ sequence binds to a heparin affinity column at pH 4.0, but does not show appreciable binding at pH 8.0.

The aggregated forms of $\alpha\beta(1-28)$ and $\alpha\beta(1-40)$ that are observed after glycosaminoglycan binding are presumed to be similar to those described by Fraser et al. (1992, J. Neurochem. 59:1531-1540), who demonstrated that $\text{SO}_4^2^-$ (5-50 mM) or heparin can cause the lateral association of pre-formed fibrils of
Aβ(11-28), Aβ(13-28) and Aβ(11-25). This group noted a decreased peptide aggregation as the pH was raised to 8.0 and no aggregation was seen when Aβ(15-28) was examined. These observations are consistent with the requirement of protonated histidines 13 and/or 14 in the S0₄⁻²⁻ interaction.

Although the present invention is not limited by any particular theory or hypothesis, one possibility is that the core protein of HSPG (and perhaps other proteoglycans) initially associates with Aβ, bringing a glycosaminoglycan chain in close apposition to residues 12-17 of the peptide. This could then alter the microenvironment around the histidine residues at positions 13 and 14, causing an elevation of the apparent pK's. Alternatively, Yates et al. (1990, J. Neurochem. 55:1624-1630) have reported decrease of pH in the AD brain, which may be sufficient in magnitude to cause an increased association of amyloid with glycosaminoglycan chains.

Recent evidence suggests that Aβ is formed within lysosomes after internalization of non-cleaved APP (Estus et al., 1992, Science 255:726-728; Haass et al., 1992, Nature 357:500-503). Although the invention is not limited by any particular theory or hypothesis, an intriguing model can be developed based on this information and the observed pH-dependent glycosaminoglycan association with the glycosaminoglycan-binding domain identified in the assays disclosed here. In this speculative scenario, proteoglycan would bind APP at the cell surface through its core protein, and some fraction of the APP with associated proteoglycan would escape secretase cleavage and be internalized into the endosomal/lysosomal pathway. Upon encountering the acidic pH of these internal organelles, a
glycosaminoglycan chain of the proteoglycan would bind tightly to the His-His-Gln-Lys domain of APP, thereby shielding the non-amyloidogenic "secretase" cleavage site at lys₁₆. Subsequent lysosomal degradation of APP, which would normally cleave the protein into non-amyloidogenic fragments, would be altered in such a way as to allow the formation of Aβ. This model would predict that an increase in proteoglycan content within the AD brain may result in an increased production of Aβ.

In summary, glycosaminoglycans bind to Aβ(13-17) at pH values below neutrality. The sulfated polysaccharides appear to bind to a consensus glycosaminoglycan-binding domain at residues 12-17 of the peptide, and one or both of two tandem histidines are likely to confer the pH dependence of this interaction. By inhibiting the interaction of Aβ with glycosaminoglycans, e.g., by using the 13-17 peptide, amyloidogenesis may be prevented or even reversed.

7. EXAMPLE: AN Aβ TETRAPEPTIDE AND A PENTAPEPTIDE AND A HEPARIN DISACCHARIDE REVERSE THE GLYCOAMINOLIGNAN MEDIATED RESISTANCE OF Aβ PEPTIDE TO DEGRADATION

The following Example provides direct evidence that Aβ peptide resists proteolytic degradation when aggregated with a proteoglycan, i.e., that proteoglycans protect aged amyloid from degeneration. Furthermore, molecules that competitively inhibit binding of Aβ peptide to glycosaminoglycans were able to reverse this resistance of Aβ to proteolytic degradation. These competitive inhibitors are Aβ(13-16), Aβ(20-24) and a heparin-derived disaccharide, α-4-deoxy-L-threo-hex-4-enopyrano-syluronic acid- [1→4] D-glucosamine-N-sulfate-6-sulfate.
7.1. MATERIALS AND METHODS

7.1.1 REAGENTS

\( \text{A}_\beta(1-40) \) was obtained from Bachem, Inc. Aged \( \text{A}_\beta(1-40) \) was solubilized 2-3 months prior to use. Fresh \( \text{A}_\beta(1-40) \) was used within 5-15 days after solubilization. Chondroitin sulfate proteoglycan (CSPG), MW 2000 kDa based on column chromatography, was purified from bovine nasal septum by dissociative guanidine HCl extraction. Papain, dextran, the heparin-derived disaccharide and other chemicals were obtained from Sigma. \( \text{A}_\beta(20-24) \) and \( \text{A}_\beta(20-24):\text{Phe}_{30}\rightarrow\text{Asp}_{20} \) were prepared by solid phase peptide synthesis as described in Section 6.1.4, supra.

7.1.2 PROTEOLYSIS RESISTANCE ASSAY

The ability of \( \text{A}_\beta \) to resist proteolysis was evaluated by testing the amount remaining after incubation with polysaccharide followed by treatment with papain. Reaction mixtures consisting of combinations of \( \text{A}_\beta(1-40) \), CSPG, dextran, and papain were prepared in 100 mM Tris, pH 6.0-7.4. The reaction mixture was incubated 45 min. Samples were incubated an additional 45 min with addition of the inhibitory molecule. Samples were then treated with papain for 8 - 18 hours. To the reaction mixture was added 2X Tris-Tricine sample buffer (8% SDS, 24% glycerol, 0.1 M Tris base, 0.1 M Tricine, 0.05% Bromophenol Blue). Aliquots were loaded on 16.5% Tris-Tricine gel. The separating gel consisted of 16.5% acrylamide, 1 M Tris base, 0.1% SDS, 13.3% glycerol, pH 8.45. The stacking gel consisted of 4% acrylamide, 1 M Tris base, 0.1% SDS, pH 8.45. After electrophoresis; gels were stained with 0.2% Coomassie brilliant blue in 45% methanol, 10% acetic acid. Gels
were scanned and $\beta$ bands were quantified using densitometry.

7.2. RESULTS
7.2.1 CSPG-MEDIATED PROTECTION OF $\beta$ PEPTIDES FROM PROTEOLYSIS

To determine whether proteoglycans protect $\beta$ from proteolysis, aged $\beta(1-40)$ was incubated with chondroitin sulfate proteoglycan (CSPG). The reaction mixture was then treated with the proteolytic enzyme papain. Control samples were run in the absence of any additives or without CSPG. The results of this experiment are shown in Fig. 13. The amount of $\beta(1-40)$ in the sample treated with papain was greatly reduced compared to the control. When incubated with CSPG prior to papain treatment, the amount of $\beta(1-40)$ recovered after proteolysis was greater than the amount recovered in the absence of CSPG. This result indicated that CSPG protects aged $\beta(1-40)$ at pH 6.0 from proteolysis.

To evaluate whether the extent of protection of $\beta(1-40)$ from proteolysis corresponds to the amount of CSPG, samples of $\beta(1-40)$ (21.5 $\mu$g) incubated with varying amounts of CSPG were treated with papain (2 $\mu$g) for 12 hours. The results are shown in Fig. 14. These results suggest a linear relationship between the amount of CSPG present in the sample and the degree of protection of the $\beta(1-40)$ from proteolysis. Based on these data, the approximate amount of CSPG required to completely protect the $\beta$ from proteolysis was calculated as 145 $\mu$g.
7.2.2 COMPETITIVE INHIBITORS OF Aβ BINDING TO CSPG REVERSE CSPG-MEDIATED PROTECTION OF Aβ FROM PROTEOLYSIS

To evaluate whether the small molecules, such as peptides that inhibit the binding of Aβ to proteoglycans, can reverse the proteoglycan/glycosaminoglycan-mediated protection of Aβ from proteolysis, potential inhibitors were added after incubation of the Aβ with CSPG, and prior to the addition of papain. The three potential inhibitors of 19 week aged Aβ(1-40)- proteoglycan/glycosaminoglycan binding that were tested were the peptide representing the heparin sulfate proteoglycan binding site of Aβ (two peptides, Aβ(13-17) and Aβ(13-16), were tested), the peptide Glu-Ala-Glu-Asp-Val, which corresponds to Aβ(20-24):Phe20 → Glu20), and a heparin disaccharide - \( \partial \text{UA}-2s-[1\rightarrow4]-\text{GlcNS}-6S \). Incubation for 45 min with any of the three inhibitor molecules was able to reverse the CSPG (30 μg) mediated protection of Aβ(1-40) (4.3 μg) from papain (4 μg) digestion for 18 hours at 37°C (Figs. 15, 16 and 17).

For the results depicted in Fig. 15, the materials, procedure, and conclusion were as follows:

Materials:
Aβ(1-40): MW 4329, LOT #ZJ209 from Bachem, Inc.
CSPG: High molecular weight CSPG was sonicated for 14 hours to generate low molecular weight material.
Peptides: Aβ(13-16) and (13-17) were synthesized.

Papain: MW 23,000 obtained from Sigma Chemical Company

Procedure:
1. Following were made in 100 mM Tris, pH 6.5:
   A. Aβ(4.3 μg)
   B. Aβ(4.3 μg)+Papain(3 μg)
   C. Aβ(4.3 μg)+CSPG(30 μg)+Papain(3 μg)
D. Aβ(4.3 μg) + CSPG(30 μg) + Aβ(13-17) + Papain (3 μg)
E. Aβ(4.3 μg) + CSPG(30 μg) + Aβ(13-16) + Papain (3 μg)

2. Aβ and CSPG were incubated together for 1 hour, following which 120 μg of Aβ(13-16) and (13-17) were added and the incubation repeated for 1 hour at room temperature.

3. Papain (3 μg) was added and the samples were incubated for 18 hours at 37° C.

4. To the reaction mixture was added 2X Tris-Tricine sample buffer (8% SDS, 24% glycerol, 0.1 M Tris base, 0.1 M Tricine, 0.05% Bromophenol blue) and samples were subjected to 16.5% Tris-Tricine SDS-polyacrylamide gel electrophoresis:
   - Separating gel: 16.5% acrylamide, 1M Tris base, 0.1% SDS, 13.3% glycerol, pH 8.45
   - Stacking gel: 4% acrylamide, 1 M Tris base, 0.1% SDS, pH 8.45
   - Gel staining: Coomassie brilliant blue (0.2% in 45% methanol and 10% acetic acid)

5. Gels were scanned and Aβ bands were quantified using a densitometer.

Conclusion

Aβ(13-16) and Aβ(13-17) disrupt Aβ-CSPG association and cause 70% increase in Aβ proteolysis. These peptides bind proteoglycans.

The Aβ(20-24: Phe_{20}→Glu_{20}) pentapeptide was synthesized based on the sequence of amyloid (residues 20-24) that are believed to bond naturally in an anti-parallel β-strand to the glycosaminoglycan-binding domain of Aβ (residues 13-17). The naturally occurring 20-24 sequence is Phe-Ala-Glu-Asp-Val, and Glu_{22} and Asp_{23} are likely to form stabilizing ion-pairs
with His$_{13}$ and His$_{14}$ of the adjacent anti-parallel strand. Substituting a negatively-charged glutamic acid for the phenylalanine at residue 20 (Phe$_{20}$ → Glu$_{20}$), to allow the negative carboxyl of the aspartic acid to ion-pair with the positively charged Lys$_{16}$, was tested to enhance binding of the pentapeptide to the glycosaminoglycan binding domain of Aβ. This should stabilize the interaction of the pentapeptide with the glycosaminoglycan-binding region at residues 13-17 as well as reduce the overall positive charge density of the 13-17 domain. The binding of the pentapeptide to residues 13-17 of existing amyloid fibrils might thus shield the positively charged amino acid residues (His$_{13}$, His$_{14}$, and Lys$_{16}$) from negatively charged glycosaminoglycan moieties, thereby reducing proteoglycan binding.

As shown in Fig. 16, the peptide Aβ (20-24: Phe$_{20}$ → Glu$_{20}$) can reverse the CSPG-mediated protection of Aβ from papain proteolysis.

7.3. DISCUSSION

This example demonstrates the evaluation of potential inhibitors of Aβ binding to glycosaminoglycans by testing ability to reverse chondroitin sulfate proteoglycan (CSPG)-mediated protection of amyloid fibrils to proteolytic degradation. Normally, the non-specific protease papain will degrade fibrillar Aβ, but prior addition of proteoglycan (e.g. CSPG or heparan sulfate proteoglycan) to the amyloid results in an inhibition of degradation. This is of significance in Alzheimer's disease, since the association of proteoglycans with amyloid within senile plaques may make the resulting structure resistant to breakdown in the brain.
Addition of Aβ(13-16) or Aβ(13-17), which contains the heparin binding domain of Aβ, reversed the CSPG-mediated protection of aged Aβ(1-40). This result is interesting since Aβ(13-17) could inhibit aggregation of Aβ(1-28) but not Aβ(1-40) with heparin or CSPG (see Section 6, supra). Since reversal of the proteolysis protection mediated by glycosaminoglycans may be more meaningful therapeutically, the inability of Aβ(13-17) to inhibit aggregation of Aβ(1-40) with heparin is less important.

Addition of the pentapeptide Aβ(20-24: Phe20-Glu20) to Aβ(1-40)-CSPG mixtures reversed the proteoglycan-mediated protection against proteolysis. Thus, Aβ(20-24 Phe20 → Glu20) appears to be effective at reducing proteoglycan-amyloid interaction. This compound may be of greater therapeutic value than the Aβ(13-17) or Aβ(13-16) compounds, since it should bind SAP and not the glycosaminoglycan chains of proteoglycans. This could make the pentapeptide a more specific drug.

In addition to the Aβ(20-24) pentapeptide, the ability of a disaccharide derived from heparin to interfere with SAP-proteoglycan binding has been examined. This disaccharide is commercially available, and has the following structure:

α-4-deoxy-L-threo-hex-4-enopyranosyluronic acid-[1 → 4] D-glucosamine-N-sulfate-6-sulfate

This molecule is similar to the Aβ(20-24) peptide in that this disaccharide is derived from the glycosaminoglycan chains that bind residues 13-17 of amyloid. Thus, this small sugar (molecular weight = 665) competes with glycosaminoglycan for binding to amyloid fibrils. As shown above, it too reverses the proteoglycan-mediated protection against proteolysis.
8. EXAMPLE: INHIBITION OF Aβ-MEDIATED COMPLEMENT ACTIVATION

While the normal activators of complement are immunoglobulins, the evidence supporting the existence of immunoglobulins in the AD brain is equivocal. This would suggest that a substance within the AD brain might bind to C1q and initiate the complement cascade.

The present example confirms the observation by Rogers et al. (1992, Proc. Natl. Acad. Sci. USA 89:10016-10020; 1992, Res. Immunol. 143(6):624-630) that the β-amyloid peptide (Aβ) is capable of specifically activating the complement cascade. This example also shows that a tetrapeptide corresponding to Aβ(13-16) and a pentapeptide derived from Aβ(20-24) are effective inhibitors of Aβ-mediated complement activation.

8.1. MATERIALS AND METHODS
8.1.1 COMPLEMENT FIXATION ASSAY

A variation of the complement fixation assay described by Palmer and Whaley (1986, in Manual of Clin. Lab. Microbiol., 3rd Ed., Rose et al. (Eds.), Am. Soc. Microbiol., pp 57-66) was utilized to assay for amyloid initiation of complement activation. Amyloid peptides were incubated overnight at 4°C in 0.2 ml of barbital buffer (pH 7.4) containing 80 µl of diluted human serum (1:100). During this incubation, peptide-mediated activation of complement results in the consumption of complement components. As a control, parallel overnight incubations of human serum were performed in the absence of added peptides. Subsequently, an aliquot (0.2 ml) of sensitized-sheep red blood cells (s-SRBC) and 0.1 ml of buffer were added to each tube and the suspension was incubated for 1 h in a 37°C water bath. Any complement
remaining from the initial overnight incubation with peptide causes lysis of the s-SRBCs, releasing hemoglobin into the medium. The suspension was centrifuged for 5 minutes at 300 x g, and the extent of complement-mediated lysis of the s-SRBCs was determined by reading the optical density of the supernatant at 410 nM. Complement activity was expressed as a percent of control samples which did not receive peptide during the initial incubation.

A solid-phase binding assay was utilized for the evaluation of Clq binding to amyloid peptide. This assay, which is similar to that recently used by Rogers et al. (1992, Proc. Natl. Acad. Sci. USA 89:10016-10020), employs Aβ which is immobilized on membranes, followed by incubation with human Clq. Briefly, Aβ is dotted onto pre-soaked PVDF membranes. The membranes are then rinsed in Tris-buffered saline pH 7.4 (TBS), followed by blocking of the membrane with 5% dry milk in TBS. After an additional rinsing in TBS, the membrane is incubated in a solution of Clq (Quidel; 10 μg/ml in TBS + 5% dry milk) for 2 h. Non-bound Clq is removed by rinsing in TBS, and bound Clq is determined by overnight incubation with rabbit anti-human Clq antibody (Quidel; 1:1000 dilution). After rinsing, the Clq antibody is visualized by a 2 h incubation with biotinylated goat anti-rabbit antibody (Quidel; 1:50 dilution) followed by color development with a Vectastain ABC Elite kit (peroxidase type).

The amount of bound Clq was quantified through densitometric analysis of the stained membranes (PDI model DNA35 densitometer).

The peptides used in these studies were Aβ(1-40) (Bachem, Inc.), Aβ(1-28) (Bachem, Inc.), or scrambled Aβ(1-40) (custom synthesized by Biosynthesis, Inc.). The scrambled peptide contains the same amino acid
composition as Aβ(1-40), with its sequence randomly assigned. Amyloid peptides were examined for activity as a function of their age in solution. In such experiments, peptides are referred to as "fresh" if they have been in aqueous solution for 1-3 days, and as "aged" if they have been in solution >25 days at 4°C. The "aged" Aβ is aggregated and of high molecular weight as judged by the sedimentation of the peptide after centrifugation at 100,000 x g for 30 min. Moreover, the aged amyloid appears to exist as β-fibrils as evidenced by its Congo Red birefringence. Ultracentrifugation reveals that the freshly prepared Aβ(1-40) solutions are not appreciably aggregated. The actual ages of the "aged" peptide solutions used in each experiment are indicated in the results. The Aβ(13-16) and Aβ(20-24) peptides were prepared by solid phase synthesis using Fmoc chemistry.

8.2. RESULTS

The major component of the AD senile plaque, β-amyloid peptide (Aβ), is capable of specifically triggering the complement cascade in vitro (Fig. 18). This activation is not initiated by control peptides, suggesting that the neuronal and axonal damage seen in AD is likely to be due in part to the formation of MAC in response to β-amyloid peptide.

The knowledge that Aβ is capable of initiating the classical complement cascade provides opportunities for specific interventions that would ameliorate complement-mediated cell damage in AD. Utilizing a solid-phase binding assay in which amyloid peptides are immobilized onto membranes and allowed to incubate with solutions containing C1q, a specific binding site residing within the first 28 residues of the 39-42 amino acid Aβ peptide was found (Fig. 19).
Based on the knowledge that the C1q binding site on immunoglobulins appears to be highly charged, and residues 13-16 of Aβ are surface exposed and bind glycosaminoglycans, it was postulated that the motif of Aβ may be responsible for C1q binding. To test this concept, a competition experiment in which a synthetic tetrapeptide consisting of residues 13-16 of Aβ was incubated at various concentrations with C1q for 1 h prior to the addition of the C1q solution to immobilized Aβ(1-28) was performed. While C1q in the absence of the tetrapeptide showed appreciable binding to Aβ(1-28), the association between C1q and Aβ(1-28) was completely inhibited by the tetrapeptide (Fig. 19). This suggests that Aβ(13-16) binds to sites on C1q and blocks subsequent interaction with Aβ(1-28), and thus implies that a C1q binding motif resides at residues 13-16 of the intact amyloid peptide.

The ability of the tetrapeptide comprising residues 13-16 of Aβ to effectively block Aβ activation of the entire complement cascade is seen in Fig. 20. Utilizing the complement fixation assay, we find that addition of a 50-fold molar excess of Aβ(13-16) results in approximately 60% inhibition of Aβ(1-40)-induced complement activation. Of particular significance is the finding that Aβ(13-16) does not inhibit the activation of complement by aggregated human immunoglobulin, indicating that amyloid must bind to a different region of C1q than immunoglobulin. This is an important observation, since immunoglobulin is the normal activator of complement and compounds based on Aβ(13-16) should not interfere with immunoglobulin-mediated complement activation throughout the body.

In addition to the Aβ(13-17) peptide, a second peptide effective to inhibit Aβ-induced complement
activation has been identified. Based on modeling of the structure assumed by Aβ in solution, residues 20-23 of the amyloid peptide are predicted to normally hydrogen-bond or bond ionically with residues 13-16 of amyloid peptide in an anti-parallel β-sheet:

\[
\begin{align*}
13 & \quad 14 & \quad 15 & \quad 16 \\
------- & \quad \text{His}--\text{His}--\text{Gln}--\text{Lys}------
\end{align*}
\]

\[
\begin{align*}
+ & \quad + & \quad + \\
- & \quad - & \quad - \\
------- & \quad \text{Asp}--\text{Glu}--\text{Ala}--\text{Phe}------
\end{align*}
\]

Residues 22 and 23 have negatively charged amino acids that neutralize the positive charge density around residues 13 and 14. To develop a peptide with the ability to bind specifically to residues 13-16 of Aβ and reduce the charge density of that motif, the pentapeptide Glu-Ala-Glu-Asp-Val was prepared. This peptide corresponds to Aβ(20-24), with the naturally occurring Phe of residue 20 substituted with a negatively charged glutamic acid. This substitution was designed to allow ion-pairing between the glutamic acid and Lys₁₆ of the full-length Aβ:

\[
\begin{align*}
12 & \quad 13 & \quad 14 & \quad 15 & \quad 16 \\
------- & \quad \text{Val}--\text{His}--\text{His}--\text{Gln}--\text{Lys}------
\end{align*}
\]

\[
\begin{align*}
+ & \quad + & \quad + \\
- & \quad - & \quad - \\
------- & \quad \text{Val}--\text{Asp}--\text{Glu}--\text{Ala}--\text{Glu}------
\end{align*}
\]

When the pentapeptide was added to the complement fixation assay, it blocked Aβ(1-40)-induced complement activation in a dose-dependent fashion (Fig. 21).
This suggests that the altered Aβ(20-24) associates with the C1q binding motif at residues 13-16 of Aβ, thereby blocking the accessibility of C1q to this domain.

Data from the C1q binding assay suggest that full-length Aβ contains a second C1q binding region within residues 25-35 (Fig. 22). Since complement activation requires that two of the six globular heads of C1q are occupied at once, it may be that C1q must bind both the 13-16 and 25-35 regions of Aβ for effective initiation of the cascade. The two classes of potential therapeutic compounds mentioned above would block binding to the 13-16 motif, and therefore disrupt one of the two required binding sites. It is possible that compounds that either resemble the binding site within residues 25-35 or interact with this site would effectively inhibit Aβ-induced activation of the complement cascade.

8.3. DISCUSSION

The results presented here confirm the observation that Aβ in amyloid plaques can activate complement.

More importantly, these results show that small peptides can inhibit Aβ-mediated complement activation. Both Aβ(13-16), a highly cationic tetrapeptide that is expected to represent a C1q binding site of Aβ, and Aβ(20-24), the putative binding site of Aβ(13-16) segment with the full-length Aβ peptide, inhibit Aβ-mediated complement activation. As demonstrated in the preceding Examples, both molecules are also effective in reversing glycosaminoglycan-mediated protection of Aβ from proteolysis. Thus these peptides, and their derivatives, are attractive therapeutic agents to
reverse the formation of amyloid plaques and reduce
the activation of complement within the brains of AD
patients.

Based on these results, disaccharides such as
heparin disaccharide are expected to inhibit
Aβ-mediated complement activation as well.

In conclusion, these results indicate that the
following groups of molecules can serve as therapeutic
agents to reduce the activation of complement in AD
brain, and thus diminish the neurological symptoms and
pathologies of the disease:

1) Specific small peptides or peptide mimetics
based on C1q binding motif within residues 13-16 or
residues 25-35 of β-amyloid peptide. These agents
associate with C1q in such a way as to block the
binding of β-amyloid;

2) Specific small peptides or peptide mimetics
that will associate with the C1q binding motif within
residues 13-16 or residues 25-35 of β-amyloid peptide.
These agents should associate with Aβ in such a way as
to block the binding of C1q; or

3) Known inhibitors of MAC attachment to
membranes, such as vitronectin, clusterin, or
protectin or peptides based on sequences from these
proteins, could be administered to AD patients. These
agents would reduce C5-C9 (MAC) attachment to neuronal
membranes, and hence would reduce the "bystander"
damage that leads to dystrophic neurites.

The present invention is not to be limited in
scope by the specific embodiments described herein.
Indeed, various modifications of the invention in
addition to those described herein will become
apparent to those skilled in the art from the
foregoing description and the accompanying figures.
Such modifications are intended to fall within the scope of the appended claims.
Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.
SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Brunden, K. et al

(ii) TITLE OF INVENTION: Inhibition of Beta Amyloid Binding to Glycosaminoglycans for Treatment of Alzheimer's Disease

(iii) NUMBER OF SEQUENCES: 7

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: To be assigned
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(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(C) REFERENCE/DOCKET NUMBER: 6739-042

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(C) TELEX: 66141 PENNIE

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln Lys
1  5 10 15
Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys Gly Ala Ile Ile
20 25 30
Gly Leu Met Val Gly Gly Val Val Ile Ala
35 40

(2) INFORMATION FOR SEQ ID NO:2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:
Lys Lys Lys Lys
1

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:
His His His His
1

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:
Asp Ala Glu Asp
1

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:
Glu Ala Glu Asp
1

(2) INFORMATION FOR SEQ ID NO:6:
(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 5 amino acids
   (B) TYPE: amino acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:
Glu Ala Glu Asp Val
1  5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 5 amino acids
   (B) TYPE: amino acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:
Asp Ala Glu Asp Val
1  5
WHAT ISCLAIMED IS:

1. A pharmaceutical composition comprising a peptide, in which the amino acid sequence of said peptide consists of X-X-N-X, in which X is a amino acid with a cationic side chain and N is a neutral amino acid; and a pharmaceutically acceptable carrier.

2. The pharmaceutical composition of claim 1 in which X is selected from the group consisting of histidine, lysine and arginine.

3. The pharmaceutical composition of claim 1 in which N is selected from the group consisting of glycine, alanine, valine, serine, threonine, asparagine, glutamine, methionine, cysteine, isoleucine, phenylalanine, and leucine.

4. The pharmaceutical composition of claim 1 in which the amino acid sequence consists of histidine-histidine-glutamine-lysine (a portion of SEQ ID NO:1).

5. A pharmaceutical composition comprising a peptide, in which the amino acid sequence of said peptide consists of X-X-N-X-Z, in which X is a amino acid with a cationic side chain, and N and Z are each independently a neutral amino acid; and a pharmaceutically acceptable carrier.

6. The pharmaceutical composition of claim 5 in which X is selected from the group consisting of histidine, lysine and arginine.

7. The pharmaceutical composition of claim 5 in which N is selected from the group consisting of
glycine, alanine, valine, serine, threonine, asparagine, glutamine, methionine, cysteine, isoleucine, phenylalanine, and leucine.

8. The pharmaceutical composition of claim 5 in which Z is selected from the group consisting of glycine, alanine, valine, leucine, isoleucine, serine, threonine, asparagine, glutamine, tryptophane, tyrosine, phenylalanine, methionine and cysteine.

9. The pharmaceutical composition of claim 5 in which the amino acid sequence consists of histidine-histidine-glutamine-lysine-leucine (a portion of SEQ ID NO:1).

10. A pharmaceutical composition comprising a peptide, in which the amino acid sequence of said peptide consists of $X_1$-N-$X_2$-$X_3$, in which at least two of $X_1$, $X_2$, and $X_3$ are independently an amino acid with an anionic side chain and the third $X$ is an amino acid with an anionic side chain or a neutral amino acid and N is independently a neutral amino acid; and a pharmaceutically acceptable carrier.

11. The pharmaceutical composition of claim 10 in which the amino acid with the anionic side chain is selected from the group consisting of aspartic acid and glutamic acid.

12. The pharmaceutical composition of claim 10 in which the non-anionic $X$ and N are independently selected from the group consisting of glycine, alanine, valine, serine, threonine, asparagine, glutamine, methionine, cysteine, isoleucine, phenylalanine, and leucine.
13. The pharmaceutical composition of claim 10 in which the amino acid sequence consists of phenylalanine-alanine-glutamic acid-aspartic acid (a portion of SEQ ID NO:1).

14. The pharmaceutical composition of claim 10 in which the amino acid sequence consists of aspartic acid-alanine-glutamic acid-aspartic acid (SEQ ID NO:4).

15. The pharmaceutical composition of claim 10 in which the amino acid sequence consists of glutamic acid-alanine-glutamic acid-aspartic acid (SEQ ID NO:5).

16. A method for treating Alzheimer's disease in a subject comprising administering a therapeutically effective amount of a peptide, in which the amino acid sequence of said peptide consists of X-X-N-X, in which X is a amino acid with a cationic side chain and N is a neutral amino acid, to a subject having Alzheimer's disease.

17. The method according to claim 16 in which X is selected from the group consisting of histidine, lysine and arginine.

18. The method according to claim 16 in which N is selected from the group consisting of glycine, alanine, valine, serine, threonine, asparagine, glutamine, methionine, cysteine, isoleucine, phenylalanine, and leucine.
19. The method according to claim 16 in which the amino acid sequence consists of histidine-histidine-glutamine-lysine (a portion of SEQ ID NO:1).

20. A method for treating Alzheimer's disease in a subject comprising administering a therapeutically effective amount of a peptide, in which the amino acid sequence of said peptide consists of X-X-N-X-Z, in which X is a amino acid with a cationic side chain, and N and Z are each independently a neutral amino acid, to a subject having Alzheimer's disease.

21. The method according to claim 20 in which X is selected from the group consisting of histidine, lysine and arginine.

22. The method according to claim 20 in which N is selected from the group consisting of glycine, alanine, valine, serine, threonine, asparagine, glutamine, methionine, cysteine, isoleucine, phenylalanine, and leucine.

23. The method according to claim 20 in which Z is selected from the group consisting of glycine, alanine, valine, leucine, isoleucine, serine, threonine, asparagine, glutamine, tryptophane, tyrosine, phenylalanine, methionine and cystine.

24. The method according to claim 20 in which the amino acid sequence consists of histidine-histidine-glutamine-lysine-leucine (a portion of SEQ ID NO:1).

25. A method for treating Alzheimer's disease in a subject comprising administering a therapeutically
effective amount of a peptide, in which the amino acid sequence of said peptide consists of $X_1-N-X_2-X_3$, in which at least two of $X_1$, $X_2$, and $X_3$ are independently an amino acid with an anionic side chain and the third $X$ is an amino acid with an anionic side chain or a neutral amino acid, and $N$ is independently a neutral amino acid, to a subject having Alzheimer's disease.

26. The method according to claim 25 in which the amino acid with the anionic side chain is selected from the group consisting of aspartic acid and glutamic acid.

27. The method according to claim 25 in which the non-anionic $X$ and $N$ are independently selected from the group consisting of glycine, alanine, valine, serine, threonine, asparagine, glutamine, methionine, cysteine, isoleucine, phenylalanine, and leucine.

28. The method according to claim 25 in which the amino acid sequence consists of phenylalanine-alanine-glutamic acid-aspartic acid (a portion of SEQ ID NO:1).

29. The method according to claim 25 in which the amino acid sequence consists of aspartic acid-alanine-glutamic acid-aspartic acid (SEQ ID NO:4).

30. The method according to claim 25 in which the amino acid sequence consists of glutamic acid-alanine-glutamic acid-aspartic acid (SEQ ID NO:5).

31. A method for treating Alzheimer's disease in a subject comprising administering a therapeutically
effective amount of an anionic disaccharide to a subject having Alzheimer's disease.

32. The method according to claim 31 in which the anionic disaccharide is a heparin-derived disaccharide.

33. The method according to claim 32 in which the heparin-derived disaccharide is α-4-deoxy-L-threo-hex-4-enopyranosyluronic acid-[1→4] D-glucosamine-N-sulfate-6-sulfate.

34. A peptide, in which the amino acid sequence of said peptide consists of X-X-N-X, in which X is an amino acid with a cationic side chain and N is a neutral amino acid.

35. The peptide of claim 34 in which X is selected from the group consisting of histidine, lysine and arginine.

36. The peptide of claim 34 in which N is selected from the group consisting of glycine, alanine, valine, serine, threonine, asparagine, glutamine, methionine, cysteine, isoleucine, phenylalanine, and leucine.

37. The peptide of claim 34 in which the amino acid sequence consists of histidine-histidine-glutamine-lysine (a portion of SEQ ID NO:1).

38. A peptide, in which the amino acid sequence of said peptide consists of X-X-N-X-Z, in which X is an amino acid with a cationic side chain, and N and Z are each independently a neutral amino acid.
39. The peptide of claim 38 in which X is selected from the group consisting of histidine, lysine and arginine.

40. The peptide of claim 38 in which N is selected from the group consisting of glycine, alanine, valine, serine, threonine, asparagine, glutamine, methionine, cysteine, isoleucine, phenylalanine, and leucine.

41. The peptide of claim 38 in which Z is selected from the group consisting of glycine, alanine, valine, leucine, isoleucine, serine, threonine, asparagine, glutamine, tryptophane, tyrosine, phenylalanine, methionine, and cysteine.

42. The peptide of claim 38 in which the amino acid sequence consists of histidine-histidine-glutamine-lysine-leucine (a portion of SEQ ID NO:1).

43. A peptide, in which the amino acid sequence of said peptide consists of $X_1-N-X_2-X_3$, in which at least two of $X_1$, $X_2$, and $X_3$ are independently an amino acid with an anionic side chain, and the third X is an amino acid with an anionic side chain or a neutral amino acid, and N is independently a neutral amino acid.

44. The peptide of claim 43 in which the amino acid with the anionic side chain is selected from the group consisting of aspartic acid and glutamic acid.

45. The peptide of claim 43 in which the non-anionic X and N are independently selected from the group consisting of glycine, alanine, valine, serine,
threonine, asparagine, glutamine, methionine, 
cysteine, isoleucine, phenylalanine, and leucine.

46. The peptide of claim 43 in which the amino 
acid sequence consists of phenylalanine-alanine-
glutamic acid-aspartic acid (a portion of 
SEQ ID NO:1).

47. The peptide of claim 43 in which the amino 
acid sequence consists of aspartic acid-alanine-
glutamic acid-aspartic acid (SEQ ID NO:4).

48. The peptide of claim 43 in which the amino 
acid sequence consists of glutamic acid-alanine-
glutamic acid-aspartic acid (SEQ ID NO:5).

49. A peptide, in which the amino acid sequence 
of said peptide consists of the sequence shown in 
Figure 1 from amino acid numbers 25-35 (a portion of 
SEQ ID NO:1).

50. A molecule comprising the amino acid 
sequence X-X-N-X, in which X is an amino acid with a 
cationic side chain and N is a neutral amino acid, 
said molecule consisting of not greater than 8 amino 
acid residues.

51. A molecule comprising the amino acid 
sequence X₁-N-X₂-X₃, in which at least two of X₁, X₂, and 
X₃ are independently an amino acid with an anionic side 
chain and the third X is an amino acid with an anionic 
side chain or a neutral amino acid and N is 
independently a neutral amino acid, said molecule 
consisting of not greater than 8 amino acid residues.
52. A molecule comprising the sequence histidine-histidine-glutamine-lysine (a portion of SEQ-ID NO. 1), said molecule consisting of not greater than 8 amino acid residues.

53. A molecule comprising the sequence glutamic acid-alanine-glutamic acid-aspartic acid (SEQ-ID NO. 5), said molecule consisting of not greater than 8 amino acid residues.

54. A method for treating Alzheimer's disease in a subject comprising administering a therapeutically effective amount of a molecule comprising an anionic disaccharide to a subject having Alzheimer's disease.

FIG. 1
FIG. 2

% SOLUBLE Aβ(1-28)

pH

40  50  60  70  80  90  100

3  4  5  6  7  8  9
FIG. 3B

Absorbance 280 nm (×10⁻³)

Aβ(1-28), pH 4.0

M

NaCl

MINUTES

0.7 0.6 0.5 0.4 0.3 0.2 0.1 0.0

0 10 20 30 40 50

2.50 1.25 0.00

0.00

0.7

0.6

0.5

0.4

0.3

0.2

0.1

0.0
FIG. 4
FIG. 5
FIG. 6

% SOLUBLE Aβ(1-40)

- TOTAL
- -GAG
- +HEPARAN SULFATE
- +CHONDROITIN SULFATE

pH 8

pH 3.5
FIG. 7A

FIG. 7B

SUBSTITUTE SHEET (RULE 26)
FIG. 9
FIG. 10
FIG. 15
A. CLASSIFICATION OF SUBJECT MATTER
IPC(6): A61K 38/00
US CL : 514/2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, CAS Online, Medline

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>US, A, 5,164,295 (KISILEVSKY ET AL.) 17 November 1992, see entire document.</td>
<td>1-54</td>
</tr>
</tbody>
</table>

Further documents are listed in the continuation of Box C. See patent family annex.

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Date of the actual completion of the international search
25 NOVEMBER 1994

Date of mailing of the international search report
06 DEC 1994

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