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(54) **Title:** A METHOD OF MODULATING BETA-AMYLOID PEPTIDE PRODUCTION

(57) **Abstract:** The present invention relates generally to a method of modulating β amyloid peptide production. More particularly, the present invention relates to a method of modulating β amyloid peptide production by downregulating cleavage of the β amyloid precursor protein to produce the β amyloid protein. The method of the present invention is useful, *inter alia*, in the treatment and/or prophylaxis of conditions characterised by aberrant, unwanted or otherwise inappropriate Aβ amyloidosis including, but not limited to, Alzheimer's disease, Down's Syndrome and cerebral amyloid angiopathy.

A method of modulating Beta-amyloid peptide production

FIELD OF THE INVENTION

The present invention relates generally to a method of modulating β amyloid peptide production. More particularly, the present invention relates to a method of modulating β amyloid peptide production by downregulating cleavage of the β amyloid precursor protein to produce the β amyloid protein. The method of the present invention is useful, *inter alia*, in the treatment and/or prophylaxis of conditions characterised by aberrant, unwanted or otherwise inappropriate Aβ amyloidosis including, but not limited to, Alzheimer's disease, Down's Syndrome and cerebral amyloid angiopathy.

BACKGROUND OF THE INVENTION

Bibliographic details of the publications referred to by author in this specification are collected alphabetically at the end of the description.

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

Alzheimer's disease is a progressive neurodegenerative disorder that is characterised by synaptic and neuronal loss (Whitehouse *et al.*, 1982, *Science 215*, 1237-1239) and the deposition of protein aggregates in the intracellular and extracellular compartments of the brain, leading to the loss of memory, cognitive disturbances and behavioural changes. The extracellular deposits or amyloid plaques consist primarily of the β-amyloid protein (Aβ) (Glenner *et al.*, 1984, *Biochem. Biophys. Res. Commun. 120*, 885-89), whereas the intracellular deposits or neurofibrillary tangles contain the microtubule-associated protein tau (Grundke-Iqbal *et al.*, 1986, *J. Biol. Chem. 261*, 6084-6089). Alzheimer's disease is

the fourth largest cause of death in the United States and affects five percent of people over age 65 and 20 percent of people over age 80. To date, there has been no established treatment developed which will prevent the onset or significantly delay the progression of Alzheimer's disease.

The most characteristic neuropathological feature of Alzheimer's disease is the deposition of β amyloid peptide (herein referred to as "Aβ") into plaques in the brain parenchyma and cerebral blood vessels leading to neuronal loss and cerebral atrophy (Terry R.D. *et al.*, 1981. Aβ is proteolytically derived from a large membrane-spanning glycoprotein known as β amyloid precursor protein (herein referred to as "APP") (Kang J. *et al,* 1987). The deposition of Aβ is believed to be closely related to the pathogenesis of Alzheimer's disease. The accumulation of Aβ in diffuse plaques is one of the earliest Alzheimer-specific neuropathological changes in Down's syndrome (Mann *et al,* 1989; Mann *et al,* 1988).

Aβ is produced from the β-amyloid precursor protein (APP) by sequential cleavage at the N- and C-termini of the Aβ domain by β- and γ-secretase, respectively (Nunan and Small, 2000, *FEBS Lett. 483*, 6-10). β-Secretase cleavage also generates a larger N-terminal fragment of APP (sAPPβ). The protease responsible for β-secretase activity in neurons has been identified as the β-site APP cleaving enzyme 1 (BACE1), also known as Asp2 or memapsin 2 (Vassar *et al.*, 1999, *Science 286*, 735-741; Sinha *et al.*, 1999, *Nature 402*, 537-540; Yan *et al.*, 1999, *Nature 402*, 533-537; Hussain *et al.*, 1999, *Mol. Cell. Neurosci. 14*, 419-427; Lin *et al.*, 2000, *Proc. Natl. Acad. Sci. U. S. A. 97*, 1456-1460). Although other substrates for BACE1 have been identified (Kitazume *et al.*, 2003, *J. Biol. Chem. 278*, 14865-14871; Kitazume *et al.*, 2005, *J. Biol. Chem. 280*, 8589-8595; Li *et al.*, 2004, *J. Biol. Chem. 279*, 10542-10550; Lichtenthaler *et al.*, 2003, *J. Biol. Chem. 278*, 48713-48719; von Arnim *et al.*, 2005, *J. Biol. Chem. 280*, 17777-17785; Wong *et al.*, 2005, *J. Biol. Chem. 280*, 23009-23017) the range of substrates may be limited *in vivo*. BACE1 knockout mice can survive to adulthood without any major phenotypic abnormality (Roberds *et al.*, 2001, *Hum. Mol. Genet. 10*, 1317-1324; Luo *et al.*, 2001, *Nat. Neurosci. 4*,

231-232; Cai *et al.*, 2001, *Nat. Neurosci. 4*, 233-234; Dominguez *et al.*, 2005, *J. Biol. Chem. 280*, 30797-30806). Therefore, decreasing Aβ load in the brain via inhibition of BACE1 activity has become an attractive strategy for Alzheimer drug development. Recently, BACE1 suppression using small interfering RNAs was shown to ameliorate the neuropathology in an AD transgenic model (Singer *et al.*, 2005, *Nat. Neurosci. (8): 1343-1349)*. Although these findings are promising, they have not, to date, led to the development of a robust means for treating Alzheimer's disease. Accordingly, there remains an ongoing need to develop means for preventing, or at least slowing, the development of Alzheimer's disease.

Heparin has been used as a research agent, in particular for affinity-purification of "heparin-binding proteins" which include numerous growth/differentiation factors, cytokines, protease inhibitor, enzymes and adhesion molecules. The heparin-binding properties of many of these proteins represents their physiological interactions with heparan sulfate (Sugahara, K. and Kitagawa, H., (2002)). Thus heparin is an effective model for evaluating interactions with heparan sulfate. By using heparin to model heparan sulfate, which occurs in the brain, in work leading up to the present invention it has been determined *in vitro* that the interaction of heparin with the BACE1 zymogen pro-domain stimulates BACE1 activity, thereby facilitating the occurrence of BACE1 mediated cleavage of the β amyloid precursor protein. This finding has therefore led to the development of a method of downregulating BACE1 mediated β amyloid precursor protein cleavage by inhibiting the *in vivo* interaction of heparan sulfate with the BACE1 pro-domain.

SUMMARY OF THE INVENTION

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

As used herein, the term "derived from" shall be taken to indicate that a particular integer or group of integers has originated from the species specified, but has not necessarily been obtained directly from the specified source. Further, as used herein the singular forms of "a", "and" and "the" include plural referents unless the context clearly dictates otherwise.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

The subject specification contains amino acid sequence information prepared using the programme PatentIn Version 3.4, presented herein after the bibliography. Each amino acid sequence is identified in the sequence listing by the numeric indicator <210> followed by the sequence identifier (eg. <210>1, <210>2, etc). The length, type of sequence (amino acid, etc.) and source organism for each sequence is indicated by information provided in the numeric indicator fields <211>, <212> and <213>, respectively. Amino acid sequences referred to in the specification are identified by the indicator SEQ ID NO: followed by the sequence identifier (eg. SEQ ID NO:1, SEQ ID NO: 2, etc). The sequence identifier referred to in the specification correlates to the information provided in numeric indicator field <400> in the sequence listing, which is followed by the sequence identifier (eg. <400>1, <400>2, etc). That is SEQ ID NO: 1 as detailed in the specification correlates to the sequence indicated as <400>1 in the sequence listing.

One aspect of the present invention is directed to a method of inhibiting or downregulating BACE1 zymogen mediated functional activity, said method comprising contacting said

BACE1 zymogen with an agent for a time and under conditions sufficient for said agent to antagonise the functional interactivity of heparin or heparan sulfate with said BACE1 zymogen pro-domain.

In another aspect there is provided a method of inhibiting or downregulating β amyloid precursor protein cleavage, said method comprising contacting the BACE1 zymogen with an agent for a time and under conditions sufficient for said agent to antagonise the functional interactivity of heparin or heparan sulfate with said BACE1 zymogen pro-domain.

In yet another aspect there is provided a method of inhibiting or downregulating BACE1 zymogen mediated functional activity, said method comprising contacting said BACE1 zymogen with an agent for a time and under conditions sufficient for said agent to antagonise the interaction of heparin or heparan sulfate with one or more basic amino acids of the BACE1 zymogen pro-domain.

In still another aspect there is provided a method of inhibiting or downregulating BACE1 zymogen mediated functional activity, said method comprising contacting said BACE1 zymogen with an agent for a time and under conditions sufficient for said agent to antagonise the interaction of heparin or heparan sulfate with one or more of H24, R27, R31, R42 and/or R45.

In a related aspect there is provided a method of inhibiting or downregulating BACE1 zymogen mediated functional activity in a mammal, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient for said agent to antagonise the functional interactivity of heparan sulfate with said BACE1 zymogen pro-domain.

In yet still another there is provided a method of inhibiting or downregulating BACE1 zymogen mediated functional activity in a mammal, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient

for said agent to antagonise the interaction of heparan sulfate with one or more basic amino acids of the BACE1 zymogen pro-domain.

In still yet another aspect there is provided a method of inhibiting or downregulating BACE1 zymogen mediated functional activity in a mammal, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient for said agent to antagonise the interaction of heparin with one or more of H24, R27, R31, R42 and/or R45.

A further aspect of the present invention is directed to a method of modulating the viability and/or functioning of Aβ amyloidogenic tissue in a subject, said method comprising administering to said subject an effective amount of an agent for a time and under conditions sufficient for said agent to antagonise the functional interactivity of heparan sulfate with the BACE1 zymogen pro-domain wherein antagonising said interaction inhibits or downregulates BACE1 zymogen mediated β amyloid precursor protein cleavage.

Another further aspect of the present invention provides a method of modulating the viability and/or functioning of Aβ amyloidogenic encephalon tissue in a subject, said method comprising administering to said subject an effective amount of an agent for a time and under conditions sufficient for said agent to antagonise the functional interactivity of heparan sulfate with the BACE1 zymogen pro-domain wherein antagonising said interaction inhibits or downregulates BACE1 zymogen mediated β amyloid precursor protein cleavage.

Yet another further aspect of the present invention is directed to a method of upregulating Aβ amyloidogenic encephalon cognitive functioning in a subject, said method comprising administering to said subject an effective amount of an agent for a time and under conditions sufficient for said agent to antagonise the functional interactivity of heparan sulfate with the BACE1 zymogen pro-domain wherein antagonising said interaction inhibits or downregulates base zymogen mediated β amyloid precursor protein cleavage.

Still another further aspect of the present invention is directed to a method for the treatment and/or prophylaxis of a condition characterised by impaired tissue viability and/or functioning, which impaired tissue viability and/or functioning is directly or indirectly induced by Aβ amyloidosis, said method comprising administering to said subject an effective amount of an agent for a time and under conditions sufficient for said agent to antagonise the functional interactivity of heparan sulfate with the BACE1 zymogen pro-domain wherein antagonising said interaction inhibits or downregulates BACE1 zymogen mediated β amyloid precursor protein cleavage.

Yet still another further aspect of the present invention provides a method for the treatment and/or prophylaxis of a condition characterised by encephalon Aβ deposition, said method comprising administering to said subject an effective amount of an agent for a time and under conditions sufficient for said agent to antagonise the functional interactivity of heparan sulfate with the BACE1 zymogen pro-domain wherein antagonising said interaction inhibits or downregulates BACE1 zymogen mediated β amyloid precursor protein cleavage.

Still yet another further aspect of the present invention provides a method for the treatment and/or prophylaxis of Alzheimer's disease in a subject, said method comprising administering to said subject an effective amount of an agent for a time and under conditions sufficient for said agent to antagonise the functional interactivity of heparan sulfate with the BACE1 zymogen pro-domain wherein antagonising said interaction inhibits or downregulates BACE1 zymogen mediated β amyloid precursor protein cleavage.

In yet another aspect, the present invention relates to the use of a modulatory agent in the manufacture of a medicament for the prophylactic and/or therapeutic treatment of a condition characterised by impaired tissue viability and/or functioning, which impaired viability and/or functioning is directly or indirectly induced by Aβ amyloidosis, wherein

said modulatory agent antagonises the functional interactivity of heparan sulfate with the BACE1 zymogen pro-domain.

In preferred embodiments of these aspects, the agent which inhibits the functional interactivity of heparin or heparan sulfate with said BACE1 zymogen pro-domain is a fragment of heparin or a pharmaceutically acceptable salt thereof. In a particularly preferred embodiment, the agent is a disaccharide of Formula (I), or pharmaceutically acceptable salt thereof.

In yet another aspect the present invention relates to a pharmaceutical composition comprising modulatory agent as hereinbefore defined and one or more pharmaceutically acceptable carriers and/or diluents. Said pharmaceutical composition may additionally comprise molecules with which it is to be co-administered. These agents are referred to as the active ingredients.

Yet another aspect of the present invention relates to modulatory agents, as hereinbefore defined, when used in the method of the present invention.

Single and three letter abbreviations used throughout the specification are defined in Table 1.

TABLE 1

Single and three letter amino acid abbreviations

Amino Acid	Three-letter Abbreviation	One-letter Symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	The	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Any residue	Xaa	X

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graphical representation of the effect of heparin on rBACE1 activity. (A) Increase in relative fluorescence (F) during rBACE1 hydrolysis of 5 μM substrate peptide in the presence of 0 μg/mL (closed circles), 1 μg/mL (open circles), 10 μg/mL (open squares) or 100 μg/mL (open triangles) heparin. Data from incubations with 0 and 1 μg/mL heparin are expressed as means ± SEM, n=4. Enzyme activity in the presence of 1 μg/mL heparin was significantly different from controls lacking heparin ($P<0.0001$, Student's *t*-test). (B) Velocity (ΔF/min) of product formation by rBACE1 as a function of substrate concentration in the absence (closed circles) or presence (open circles) of 1 μg/mL heparin. Data for 1-30 μM substrate peptide are expressed as means ± SEM, n=2-9. The velocity of the reaction in the presence of 1 μg/mL heparin was significantly different from controls ($P<0.0001$, Student's *t*-test). Curves shown are calculated from a best fit of the data to the Michaelis-Menten equation. In the absence or presence of heparin, respectively, the V_{max} was calculated as 15.9±0.9 ΔF/min and 30.3±1.6 ΔF/min SEM, and the K_M was 11.4±1.4 μM and 6.5±0.9 μM SEM. (C) Velocity (ΔF/min) of product formation by rBACE1 using 10 μM substrate peptide in the absence (control) or presence of 4 μg/mL apoE (apoE), 1 μg/mL heparin (Hep) and 1 μg/mL heparin with 4 μg/mL apoE (Hep + apoE). Similar results were obtained in three separate experiments. The figure shows data expressed as means ± range, n=2, for one representative experiment.

Figure 2 is a graphical representation of the time course of activation of rBACE1 by heparin. (A-C) Increase in F during rBACE1 hydrolysis at different concentrations of substrate peptide as indicated, in the absence (closed circles) or presence (open circles) of 1 μg/mL heparin. Data are expressed as means ± SEM, n=2-6. Enzyme activity in the presence of heparin was significantly different from controls lacking heparin at all the substrate concentrations tested ($P<0.0001$ for 0.5 and 20 μM substrate and $P<0.01$ for 1 μM substrate, Student's *t*-test). The phase of stimulation (stim.) and inhibition is shown.

Figure 3 is an image of the effect of heparin on autocatalytic cleavage of rBACE1. (A)

Silver-stained gel after SDS-PAGE. rBACE1 (1.6 μg in 100 μL, pH 4, 5 % DMSO) was incubated in the absence (control) or presence of heparin (2 μg/mL) for 15 min at 37 °C and an aliquot (25 μL) from each incubation was analysed. (B-E) Western blot analysis of rBACE1 using the indicated antibodies. For each incubation, one representative blot of 2-3 separate experiments is shown. (B) rBACE1 (0.8 μg) was incubated in a volume of 100 μL 0.1 M sodium acetate buffer (pH 4, 5 % DMSO) at 37 °C and aliquots of 10 μL were analysed after 0 to 60 min incubation in the presence of 1 μg/mL heparin. (C) Incubation of rBACE1 as in B, in the presence of 0 μg/mL (control), 1 μg/mL or 100 μg/mL heparin. The immunoblots were probed, stripped and reprobed using the antibodies in the following order: anti-His, EE-17 and anti-proBACE1. (D) Incubation of rBACE1 as in B except that 0.1 M sodium phosphate buffer (pH 7) was used. Incubations contained 0 μg/mL (control), 1 μg/mL or 100 μg/mL heparin. (E) rBACE1 (0.8 μg in 100 μL, pH 4, 5 % DMSO) was incubated with 5 μM substrate peptide in the absence (control) or presence of 1 μg/mL heparin at 37 °C. Aliquots of 10 μL were analysed after 0 to 60 minutes. The immunoblots were probed, stripped and reprobed using the antibodies in the following order: anti-His, anti-proBACE1 and EE-17. (F-G) Quantification of the immunoreactivity of the 70-73 kDa protein to the anti-proBACE1 (open circles), the EE-17 (closed circles) and the anti-His (closed squares) antibodies. rBACE1 was incubated for 0 to 60 min with heparin (1 μg/mL) in the absence (F) or presence (G) of substrate peptide (5 μM). For each antibody, the intensity of the signals obtained on the film was measured and the data are expressed relative to the intensity at time 0. (A-G) Time 0 represents 10 s after start of the reaction. * 70-73 kDa form, **65-68 kDa form, *** 62 kDa form, **** 40-50 kDa forms.

Figure 4 is an image depicting that the pro-enzyme form of rBACE1 interacts strongly with heparin. (A) Western blot analysis of an aliquot (20 μL) from the fractions eluted from a heparin affinity column with increasing concentrations of NaCl as indicated. rBACE1 (15 μg) was applied to the column. Lane 1, 100 ng rBACE1. The immunoblot was first incubated with anti-proBACE1 antibody and then stripped and reprobed with the EE-17 antibody. (B) Activity of rBACE1 during hydrolysis of 10 μM substrate peptide in

the enzyme fraction recovered in the 1.2 M NaCl wash. Incubations were performed in the absence (closed circles) or presence (open circles) of 1 μg/mL heparin in 0.1 M sodium acetate buffer (pH 4). Similar results were obtained in 2 separate experiments.

Figure 5 is a graphical representation depicting that a synthetic peptide homologous to the pro-sequence of BACE1 binds heparin. RP-HPLC analysis of fractions eluted with increasing concentrations of NaCl as indicated during heparin affinity chromatography of the synthetic pro-peptide. The pro-peptide (retention time = 28.4 min, arrow) was recovered almost exclusively in the 0.5 M fraction.

Figure 6 is an image depicting the effect of heparin on mature rBACE1. (A) BACE1 activity (F) during hydrolysis of 10 μM substrate peptide by 100 ng rBACE1 (upper panel) or by a pro-enzyme depleted supernatant fraction obtained after immunoprecipitation of rBACE1 with the anti-proBACE1 antibody (lower panel). Incubations were performed in the absence (closed circles) or presence (open circles) of 1 μg/mL heparin in a volume of 100 μL at pH 4. (B) Western blot analysis of rBACE1. Lane 1, 100 ng rBACE1; lane 2, supernatant fraction; lane 3-6, analysis of aliquots (15 μL) taken after the enzyme activity assay in A; lane 3, supernatant fraction; lane 4, supernatant fraction incubated with heparin (1 μg/mL); lane 5, rBACE1 and lane 6, rBACE1 incubated with heparin (1 μg/mL). The immunoblot was first incubated with anti-proBACE1 antibody and then stripped and reprobed with the EE-17 antibody. Similar results were obtained in 2 separate experiments.

Figure 7 is a graphical representation of MALDI-TOF MS analysis of autocatalytic cleavage products of rBACE1. (A) MALDI-TOF MS spectra showing the percentage intensity against mass/charge (m/z) ratio in the range of 1000 to 3500 (A) and 3500 to 15 000 (B) obtained from a sample of rBACE1 which had been activated by heparin. Inset in A shows the product ion spectra (MSMS) of the peptides corresponding to peak a and b. (C) The observed masses of peaks a-d were consistent with the theoretical monoisotopic masses of the peptides shown. (D) Schematic illustration of the amino acid sequence and reported functional domains of BACE1(1-460) (Vassar *et al.*, 1999, *supra*).

Abbreviations: SP, signal peptide; PRO, pro-domain; CAT, catalytic domain. Asterisks denote catalytically active aspartates and numbers indicate amino acid position. Arrows show the putative heparin-induced cleavage sites as identified from the MS and tandem MS analysis. The figure also shows the catalytically active aspartate-93 (D) in bold. Representation of BACE1 not drawn to scale.

Figure 8 is a schematic representation of the 3D structure of mature (pro-sequence cleaved) BACE1 showing the location of heparin stimulated autocatalytic cleavage sites and the location of the two catalytic aspartate residues (D).

Figure 9 is a graphical representation of the effect of heparin and heparin disaccharide I-S (H9267) on the rate of BACE1 cleavage of a substrate peptide. The concentration of heparin was 1μg/ml. The concentration of H9267 was 10μg/ml.

Figures 10A-10D graphically depict the effect of increasing the level of sulfation of heparin disaccharides on BACE1 activity. BACE1 was incubated with a quenched fluorogenic substrate and 0, 1, 10 or 100 μg/ml heparin disaccharide, in the absence (white bar) or presence (black bar) of 1 μg/ml heparin. Each reaction was measured over 180 minutes using a fluorescent plate reader. Figure 10A depicts the effect of non-sulfated heparin disaccharide (OD-HD, heparin disaccharide IV-A). Figure 10B depicts the effect of mono-sulfated heparin disaccharide (IS-HD, heparin disaccharide III-H). Figure 10C depicts the effect of di-(N2)-sulfated heparin disaccharide (2S2-HD, heparin disaccharide III-S). Figure 10D depicts the effect of tri-(N,2,6)-sulfated heparin disaccharide (3S-HD, heparin disaccharide I-S).

Figures 11A-11B graphically depict percentage inhibition of heparin-induced activation of BACE1 by the disaccharide 3S-HD (Figure 11A) and the tetrasaccharide 3S-HT (Figure 11B). IC_{50} values of 44.1 μM and 0.80 μM respectively were calculated.

Figures 12A-12B graphically depict the effect of 3S-HT on sAPPα (Figure 12A) and

Aβ40 and Aβ42 (Figure 12B) in hAPP-CHO cells. Levels of sAPPα were measured by Western blotting. Figure 12B shows 3S-HT inhibits Aβ40 and Aβ42 production in hAPP-CHO cells. In contrast, sAPPα production is stimulated by 3S-HT.

Figure 13 graphically depicts the effect of 3S-HT i.p. treatment to decrease amyloid load (Thioflavin S staining) in a transgenic mouse model of AD (Tg 2576). Mice were injected with 10μg 3S-HT 3x per week from 8 months to 12 months of age.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is predicated, in part, on the determination that BACE1 zymogen mediated functional activity, in particular BACE1 zymogen mediated β amyloid precursor protein cleavage, is modulatable via the interaction of heparin or heparan sulfate with the pro-domain region of the BACE1 zymogen. Accordingly, this finding has most particularly facilitated the development of means for downregulating BACE1 mediated functional activity via the use of agents which inhibit the functional interactivity of heparin or heparan sulfate with the BACE1 zymogen pro-domain. This determination now permits the rational design of therapeutic and/or prophylactic methods for treating, either adjunctively or otherwise, conditions characterised by aberrant or otherwise unwanted Aβ amyloidosis such as occurs in Alzheimer's disease and Down's Syndrome.

Accordingly, one aspect of the present invention is directed to a method of inhibiting or downregulating BACE1 zymogen mediated functional activity, said method comprising contacting said BACE1 zymogen with an agent for a time and under conditions sufficient for said agent to antagonise the functional interactivity of heparin or heparan sulfate with said BACE1 zymogen pro-domain.

Reference to "BACE1 zymogen" should be understood as a reference to all forms of BACE1 which comprise the pro-domain and protease domain and including, for example, any isoforms which arise from alternative splicing of BACE1 zymogen mRNA or allelic or polymorphic variants of the BACE1 zymogen. It should also be understood that reference to the subject zymogen also includes reference to any form which includes amino acid domains in addition to the pro-domain and the protease domain. Accordingly, said BACE1 zymogen form may or may not also include one or more of the signal peptide, transmembrane domain or cytoplasmic tail. Without limiting the present invention to any one theory or mode of action, BACE1 belongs to the family of aspartic proteases, although it differs from other family members by being membrane anchored (Yan *et al.*, 1999, *supra*). Full-length BACE1 contains 501 amino acid residues including a signal peptide sequence and a 24 amino acid residue pro-domain. The protease domain is located

between residues 46-460 and is followed by a transmembrane domain and a short cytoplasmic tail. Because the catalytic ectodomain of BACE1 can be shed from the membrane, the enzyme also exists in a soluble form (Benjannet *et al.*, 2001, *J. Biol. Chem. 276*, 10879-10887; Hussain *et al.*, 2003, *J. Biol. Chem. 278*, 36264-36268).

During its maturation in the secretory pathway, BACE1 1 undergoes complex N-linked glycosylation (Charlwood *et al.*, 2001, *J. Biol. Chem. 276*, 16739-16748; Haniu *et al.*, *J. Biol. Chem. 275*, 21099-21106). The pro-domain, which is important for the exit of proBACE1 from the endoplasmic reticulum (Banjannet *et al.*, 2001, *supra*) and for proper folding of the protease domain (Shi *et al.*, 2001, *J. Biol. Chem. 276*, 10366-10373), is removed, possibly by furin or other members of the pro-protein convertase (PC) family (Bennett *et al.*, 2000, *J. Biol. Chem. 275*, 37712-37717; Creemers *et al.*, 2001, *J. Biol. Chem. 276*, 4211-4217). Removal of the pro-sequence increases enzyme activity (Shi *et al.*, 2001, *supra*). However, unlike many other aspartic proteases, the pro-domain does not completely suppress the activity of the zymogen.

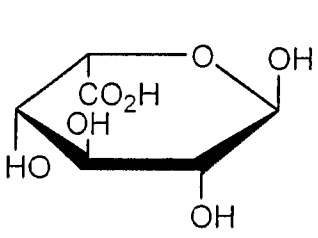
In terms of the BACE1 zymogen which is referred to in the context of the present invention, it should be understood that the amino acid numbering system which is utilised herein is based on the numbering as allocated to the 501 amino acid full-length form of this zymogen. Accordingly, the 24 amino acid pro-domain is defined by amino acids 22-45, as follows:

TQHGIRLPLRSGLGGAPLGLRLPR (SEQ ID NO:1)

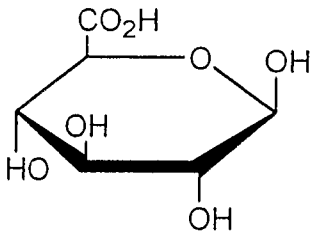
and wherein residue motif TQH corresponds to residue numbers 22, 23 and 24, respectively, and the residue RLPR corresponds to residue numbers 42, 43, 44 and 45, respectively. It should also be understood that, for continuity of reference, this system of numbering is used in this specification irrespective of whether the BACE1 zymogen in issue actually includes the 21 amino acid signal peptide region.

As detailed hereinbefore, it has been determined *in vitro* that heparin promotes β secretase

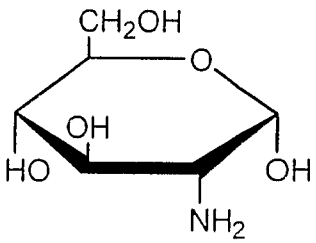
cleavage of β amyloid precursor protein by upregulating BACE1 activity via its interaction with the BACE1 zymogen pro-domain. *In vivo*, it is heparan sulfate which upregulates BACE1 activity via its interaction with the BACE1 zymogen pro-domain. Although heparin is a molecule which is not generally found *in vivo*, it has been determined that molecules which bind to heparin will also interact with endogenous heparan sulphate. Accordingly, heparin is therefore a highly useful molecule in terms of establishing screening assays to identify molecules which inhibit the interaction of BACE1 zymogen pro-domain either with endogenous heparan sulphate or with exogeneously introduced heparin. Heparin is a linear highly sulfated mucopolysaccharide comprising alternating residues of uronic acid, either as D-glucuronic acid or its epimeric L-iduronic acid, and D-glucosamine. The resulting uronic acid→glucosamine disaccharide units constitute the "monomer" units of the polymer. It is isolated from animal sources, usually porcine or bovine intestines or lung.



iduronic acid



glucuronic acid



glucosamine

Unfractionated heparin is a heterogenous mixture of polysaccharide chains. Complexity arises not only from the variation in chain length - the size of an individual chain length can reach 100 kDa but is typically from about 3,000 to about 40,000 Da with an average of about 12-15,000 Da - but also from the pattern and extent of O-sulfation at C2 of the uronic acid residue and C3 and C6 of the glucosamine residue. In addition, the glucosamine residues may also be N-acetylated or N-sulfated. The closely related heparan sulfate (HS) is a less sulfated analogue of heparin, the proportion of N-sulfation being typically below 50% while in heparin it is usually 70% or higher.

The "pro-domain" with which heparin has been shown to interact has been hereinbefore defined as corresponding to amino acids 22-45 of the full length BACE1 zymogen. Accordingly, reference to BACE1 zymogen "pro-domain" should be understood as a

reference to this sequence of amino acids or functional fragment thereof. By antagonising the interaction of heparin or heparan sulfate with this pro-domain, BACE1 zymogen mediated functional activity is downregulated. To this end, reference to antagonising the "functional interactivity" of heparin or heparan sulfate with the BACE1 zymogen pro-domain should be understood as a reference to antagonising the subject interaction such that heparin or heparan sulfate induced BACE1 zymogen activation is either minimised or entirely abrogated. In a preferred embodiment, this is achieved by entirely preventing the interaction of heparin or heparan sulfate with the BACE1 zymogen pro-domain by binding an agent to the pro-domain which hinders heparin or heparan sulfate binding. However, it should also be understood to extend to the situation in which the subject agent does not necessarily entirely prevent heparin or heparan sulfate from binding with the pro-domain, but only partially antagonises binding of the heparin molecule such that whatever degree of interaction may occur between heparin or heparan sulfate and the pro-domain is nevertheless insufficient to activate the BACE1 zymogen to the extent that is normally induced by heparin.

Accordingly, reference to antagonising "functional interactivity" is a reference to either entirely preventing binding of heparin or heparan sulfate to the pro-domain or else to sufficiently disrupting the binding event such that the functional outcome of activating the BACE1 zymogen is either abrogated or at least downregulated. Although it is preferable that heparin or heparan sulfate induced BACE1 zymogen activity is entirely abrogated, it would be appreciated that even downregulating the extent or degree of activation can nevertheless produce extremely valuable therapeutic outcomes in terms of minimising Aβ formation and, thereby, at least minimising, even if not entirely preventing, Aβ amyloidosis.

Without limiting the present invention to any one theory or mode of action, the induction of BACE1 activation, and its subsequent clearance is relatively complex. For example, the zymogen form of BACE1 exhibits a basal level of activity which can be upregulated subsequently to heparin or heparan sulfate binding to the pro-domain. However, mature BACE1, in which the pro-domain has been cleared off, similarly exhibits increased activity

relative to the zymogen form. Ultimately, however, the enzymatic activity of both these forms of the enzyme is lost by virtue of the autocatalytic cleavage events which the protease domain undergoes. Accordingly, it would be appreciated that even if the method of the present invention entirely abrogates heparin induced activation of the BACE1 zymogen, there may nevertheless still occur some level of β-secretase cleavage of β amyloid precursor protein by virtue of the functioning of any mature BACE1 which is present or the basal activity of the BACE1 zymogen. However, in the absence of the functional contribution of heparin-activated BACE1 zymogen, the level of β-secretase functioning is significantly reduced. Still further, when considered together with the BACE1 protease autocatalysis events which effectively clear functional BACE1 relatively quickly, the level of Aβ production which can occur in an individual undergoing treatment according to the method of the present invention is, relatively speaking, significantly lower than would occur in an individual who is not undergoing this treatment.

Although the method of the present invention is particularly useful in the context of downregulating BACE1 mediated cleavage of β amyloid precursor protein to form Aβ, it should be understood that BACE1 may also exhibit other functional roles which are negatively impacted upon by downregulating heparin or heparan sulfate induced activation of the BACE1 zymogen. Accordingly, reference to "BACE1 zymogen functional activity" should be understood as a reference to any functional outcome which can be either directly or indirectly induced by heparin or heparan sulfate-activated BACE1 zymogen. For example, and without limiting the present invention in any way, the inhibition of heparan sulfate binding to BACE1 is thought to:

(i) downregulate BACE1 activation;
(ii) downregulate BACE1 trafficking; and/or
(iii) increase BACE1 degradation.

Reference to "functional activity" should therefore be understood to extend to any of these direct functional events, in addition to downstream functional outcomes, such as the downregulation of β amyloid precursor protein cleavage.

Preferably, said functional activity is β amyloid precursor protein cleavage.

According to this preferred embodiment there is provided a method of inhibiting or downregulating β amyloid precursor protein cleavage, said method comprising contacting the BACE1 zymogen with an agent for a time and under conditions sufficient for said agent to antagonise the functional interactivity of heparin or heparan sulfate with said BACE1 zymogen pro-domain.

Preferably, said downregulated β amyloid precursor protein cleavage corresponds to downregulated Aβ production.

The interaction of heparin or heparan sulfate with the BACE1 zymogen pro-domain is though to occur at the level of the interaction of the sulfate groups of heparin or heparan sulfate with the basic amino acids of the pro-domain. The BACE1 zymogen pro-domain is characterised by 5 positively charged amino acid residues, these being H24, R27, R31, R42 and R45. Accordingly, the agent of the present invention preferably antagonises the interaction of heparin or heparan sulfate with one or more of amino acid residues 22-45 of BACE1, more preferably one or more of the basic amino acids of amino acid residues 22-45 of BACE1 and most preferably one or more of H24, R27, R31, R42 and/or R45.

The present invention therefore preferably provides a method of inhibiting or downregulating BACE1 zymogen mediated functional activity, said method comprising contacting said BACE1 zymogen with an agent for a time and under conditions sufficient for said agent to antagonise the interaction of heparin or heparan sulfate with one or more basic amino acids of the BACE1 zymogen pro-domain.

Still more preferably, there is provided a method of inhibiting or downregulating BACE1 zymogen mediated functional activity, said method comprising contacting said BACE1 zymogen with an agent for a time and under conditions sufficient for said agent to antagonise the interaction of heparin or heparan sulfate with one or more of H24, R27,

R31, R42 and/or R45.

Preferably, said BACE1 zymogen mediated functional activity is β amyloid precursor protein cleavage. Still more preferably, said cleavage corresponds to downregulated Aβ production.

It should be understood that the method of the present invention has application both *in vitro* and *in vivo* where heparin can be used *in vitro* to model the *in vivo* occurring heparan sulfate. Although the preferred application of this method is *in vivo* in the context of the treatment of a patient, it should be understood that *in vitro* application is also contemplated. For example, the method of the present invention is particularly useful when applied as an *in vitro* assay for screening for the modulatory agents which are hereinafter discussed in more detail.

Accordingly, in a related aspect there is provided a method of inhibiting or downregulating BACE1 zymogen mediated functional activity in a mammal, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient for said agent to antagonise the functional interactivity of heparan sulfate with said BACE1 zymogen pro-domain.

More particularly, there is provided a method of inhibiting or downregulating BACE1 zymogen mediated functional activity in a mammal, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient for said agent to antagonise the interaction of heparan sulfate with one or more basic amino acids of the BACE1 zymogen pro-domain.

Still more particularly, there is provided a method of inhibiting or downregulating BACE1 zymogen mediated functional activity in a mammal, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient for said agent to antagonise the interaction of heparan sulfate with one or more of H24, R27, R31, R42 and/or R45.

Preferably, said BACE1 zymogen mediated functional activity is β amyloid precursor protein cleavage. Still more preferably, said cleavage corresponds to downregulated Aβ production.

Reference to "agent" should be understood as a reference to any proteinaceous or non-proteinaceous molecule which downregulates the interaction of heparin or heparan sulfate with the BACE1 zymogen pro-domain. The subject agent may be linked, bound or otherwise associated with any proteinaceous or non-proteinaceous molecule. For example, it may be associated with a molecule which permits targeting to a specific tissue, such as the brain.

Said proteinaceous molecule may be derived from natural, recombinant or synthetic sources including fusion proteins or following, for example, natural product screening. Said non-proteinaceous molecule may be derived from natural sources, such as for example natural product screening or may be chemically synthesised. For example, the present invention contemplates chemical analogues of the heparin fragments which are exemplified later in this document. The agent is preferably an antagonist which is capable of blocking, inhibiting or otherwise preventing the interaction of heparin or heparan sulfate with the pro-domain. Antagonists include antibodies (such as monoclonal and polyclonal antibodies) specific for all or part of the pro-domain. Antagonists also include small molecules which are designed to interact with the pro-domain, in particular the pro-domain basic residues, thereby functioning as a competitive inhibitor of heparin or heparan sulfate binding.

Screening for the agents hereinbefore defined can be achieved by any one of several suitable methods including, but in no way limited to, contacting a BACE1 zymogen pro-domain or functional fragment thereof with an agent and screening for the interaction of these molecules or for modified functionality, such as modified activation potential in the presence of heparin or heparan sulfate. Detecting such outcomes can be achieved utilising techniques such as Western blotting, electrophoretic mobility shift assays and/or the

readout of reporters of BACE1 activity such as luciferases, CAT, APP assays and the like.

Accordingly, the subject of detection could be a downstream BACE1 regulatory target (for example, β amyloid precursor protein), rather than BACE1 itself. Yet another example includes BACE1 pro-domain heparin/heparan sulfate binding sites ligated to a minimal reporter. In another example, modulation of BACE1 pro-domain functionality can be detected by screening for the modulation of APP cleavage to form Aβ. This is an example of an indirect system, where modulation of heparin or heparan sulfate interactivity , *per se*, is not the subject of detection. Rather, modulation of the level of the heparin or heparan sulfate induced BACE1 zymogen β amyloid precursor protein cleavage activity is monitored. These methods provide a mechanism for performing high throughput screening of putative modulatory agents such as the proteinaceous or non-proteinaceous agents comprising synthetic, combinatorial, chemical and natural libraries.

The agents which are utilised in accordance with the method of the present invention may take any suitable form. For example, proteinaceous agents may be glycosylated or unglycosylated, phosphorylated or dephosphorylated to various degrees and/or may contain a range of other molecules fused, linked, bound or otherwise associated with the proteins such as amino acids, lipids, carbohydrates or other peptides, polypeptides or proteins. Similarly, the subject non-proteinaceous molecules may also take any suitable form. Both the proteinaceous and non-proteinaceous agents herein described may be linked, bound otherwise associated with any other proteinaceous or non-proteinaceous molecules. For example, in one embodiment of the present invention said agent is associated with a molecule which permits its targeting to a localised region, such as a specific tissue.

The subject agent may be derived from any source such as being chemically synthesised or identified via screening processes such as natural product screening. For example agents can be designed and/or identified utilising well known methods such as combinatorial chemistry or high throughput screening of recombinant libraries or following natural product screening.

For example, libraries containing small organic molecules may be screened, wherein organic molecules having a large number of specific parent group substitutions are used. A general synthetic scheme may follow published methods (eg., Bunin *et al.* (1994) *Proc. Natl. Acad. Sci. USA, 91:*4708-4712; DeWitt *et al.* (1993) *Proc. Natl. Acad. Sci. USA,* **90**:6909-6913). Briefly, at each successive synthetic step, one of a plurality of different selected substituents is added to each of a selected subset of tubes in an array, with the selection of tube subsets being such as to generate all possible permutation of the different substituents employed in producing the library. One suitable permutation strategy is outlined in US. Patent No. 5,763,263.

There is currently widespread interest in using combinational libraries of random organic molecules to search for biologically active compounds (see for example U.S. Patent No. 5,763,263). Ligands discovered by screening libraries of this type may be useful in mimicking or blocking natural ligands or interfering with the naturally occurring ligands of a biological target. In the present context, for example, they may be used as a starting point for developing BACE1 pro-domain antagonists. The pro-domain or a relevant part thereof may, according to the present invention, be used in combination libraries formed by various solid-phase or solution-phase synthetic methods (see for example U.S. Patent No. 5,763,263 and references cited therein). By use of techniques, such as that disclosed in U.S. Patent No. 5,753,187, millions of new chemical and/or biological compounds may be routinely screened in less than a few weeks. Of the large number of compounds identified, only those exhibiting appropriate biological activity are further analysed.

With respect to high throughput library screening methods, oligomeric or small-molecule library compounds capable of interacting specifically with a selected biological agent, such as a biomolecule, a macromolecule complex, or cell, are screened utilising a combinational library device which is easily chosen by the person of skill in the art from the range of well-known methods, such as those described above. In such a method, each member of the library is screened for its ability to interact specifically with the selected agent. In practising the method, a biological agent is drawn into compound-containing tubes and

allowed to interact with the individual library compound in each tube. The interaction is designed to produce a detectable signal that can be used to monitor the presence of the desired interaction. Preferably, the biological agent is present in an aqueous solution and further conditions are adapted depending on the desired interaction. Detection may be performed for example by any well-known functional or non-functional based method for the detection of substances.

In particular embodiments of the invention, the agent which inhibits the functional interactivity of heparin or heparan sulfate with said BACE1 zymogen pro-domain is a fragment of heparin.

As used herein a "fragment of heparin" refers to an oligosaccharide containing alternating uronic acid and glucosamine residues. Exemplary oligosaccharides are up to about 5 kDa in size. Alternatively, the fragment of heparin can be defined by the number of saccharide units. Thus, further exemplary oligosaccharides have up to about 8 disaccharide (ie. 16 monosaccharide) units. In further embodiments, oligosaccharides have up to about 6 disaccharide (ie. 12 monosaccharide) units. Other oligosaccharides are decasaccharides (10 monosaccharide or 5 disaccharide units), octasaccharides (8 monosaccharides or 4 disaccharides), hexasaccharides (6 monosaccharides or 3 disaccharides), tetrasaccharides (4 monosaccharides or 2 disaccharides) and disaccharides. The agent may consist of essentially a single (homogeneous, e.g. about 100%) oligosaccharide entity or comprise a heterogeneous mixture of oligosaccharide entities, having variation in the pattern of O- and N-sulfation and N-acetylation, and/or chain length. Where the product is a mixture of oligosaccharides, particularly with variation in chain length, reference to the number of disaccharide and/or monosaccharide units, or molecular weight, is taken to refer to a size or weight average as appropriate. In certain embodiments, reference to an oligosaccharide by unit length (e.g. disaccharide, tetrasaccharide etc.) refers to a substantially pure chain length, e.g. at least 70%, such as at least 75, 80, 95, 97 or 99% of the nominated chain length. Similarly a nominated pattern of sulfation refers to an oligosaccharide product having at least 70, 75, 80, 85, 90, 95, 97 or 99% of the nominated sulfation pattern. The terms "uronic acid residue" and "glucosamine residue" include cyclic and acyclic forms

and also include the modified forms thereof such as resulting from depolymerization treatment, eg "uronic acid residue" includes the C4-C5 unsaturated uronic acid resulting from heparinase digestion and "glucosamine residue" includes the anhydromannose (2,5-anhydro-D-mannose) residue resulting from nitrous acid polymerization or the corresponding alditol formed by subsequent reduction.

Such a fragment may be a product obtained by depolymerization or chromatographic fractionation of heparin or a derivative thereof. As used herein, a derivative of heparin includes heparan sulfate and chemically modified forms of heparin. Chemically modified forms of heparin are available from commercial sources such as Neoparin Inc. CA, USA . These include:

Oversulfated Heparin, wherein all the primary hydroxyls in glucosamine residues and a large proportion of secondary hydroxyl groups in disaccharide units have been substituted by O-sulfate esters;

Carboxyl-Reduced Heparin, in which the carboxyl group of uronic acid residues of heparin have been reduced to alcohols. The disaccharide units in the derivative are either glucose - glucosamine or idose - glucosamine;

Periodate-Oxidized Heparin, in which all unsulfated uronic acid residues of heparin are oxidized by periodic acid. After periodate oxidation the newly formed aldehydes are reduced to primary alcohols;

Fully De-O-Sulfated Heparin, in which all O-sulfate esters of heparin have been removed without changing the backbone structure;

2-O-Desulfated Heparin, in which only the O-sulfate groups on C-2 of uronic acid residues have been removed;

6-O-Desulfated Heparin, in which most of O-sulfate groups on C-6 of glucosamine

residues have been removed while most of the 2-O-sulfate / 3-O-sulfate groups are remain intact;

Fully N-Acetylated Heparin, prepared by N-acetylation of De-N-Sulfated Heparin. All disaccharide units in the derivative are uronic acid -> N-acetyl-glucosamine units;

Fully N-Sulfated Heparin, made by N-sulfation of De-N-Acetylated Heparin. The sequence of the disaccharide units in the derivative are exclusively uronic acid -> N-sulfate-glucosamine;

De-N-Sulfated Heparin, in which N-sulfate groups of the N-sulfated-glucosamine residues of heparin are removed. The altered disaccharide residues in the derivative become uronic acid -> glucosamine, while the unmodified disaccharide units remain as uronic acid -> N-acetyl-glucosamine; and

De-N-Acetylated Heparin, in which all N-acetyl groups of the N-acetyl-glucosamine residues of heparin have been depleted. The altered disaccharide unit in the derivative becomes uronic acid -> glucosamine while the unmodified disaccharide unit remains as uronic acid -> N-sulfate-glucosamine.

Depolymerization of heparin or its derivatives can be carried out by any suitable means known in the art, such as enzymatic digestion, chemical treatment or irradiation as appropriate. The depolymerized products may be separated or purified by well-known methods such as various chromatographic techniques or high resolution filtration. It will be appreciated that although oligosaccharide fragments may be obtained by depolymerization, synthetic oligosaccharides (see for example, Ferro D.R. *et al* (1990), G. Jaurand, *et al* (1994) and Chiba, T. *et al*, (1998) are also contemplated by the present invention.

Enzymatic depolymerisation (see for example, Silva, M.E. and Dietrich, C.P. (1974); Nader, H.B., *et al* (1989); Nader, H.B., *et al*, (1990) Ernst, S. *et al.* (1995) and McLean,

M.W. *et al.* (1985)) may be carried out by digestion by various heparinases. Heparinases I, II and III are isolable from *Flavobacterium heparinum* and selectively cleave sulfate glycans containing α(1-4) glycosidic linkages between the glucosamine and uronic acid residues. Generally, the cleavage proceeds via an elimination reaction resulting in the formation of oligosaccharide fragments containing unsaturated uronic acid residues. The three forms of heparinase have varying substrate specificities.

Heparinase I cleaves heparin and heparan sulfate (relative activity about 3:1) at the linkages between glucosamines and O-sulfated iduronic acids, yielding mainly disaccharides.

Heparinase II cleaves heparan sulfate, and to a lesser extent heparin (relative activity about 2:1) at the 1-4 linkages between glucosamines and uronic acid residues (both glucuronic and iduronic), yielding mainly disaccharides. The lyase activity of Heparinase II had been previously characterised as "heparitinase II" (Silva, M.E. and Dietrich, C.P., (1974).

Heparinase III cleaves at the 1-4 linkages between hexosamine and glucuronic acid residues in heparin sulfate, yielding mainly disaccharides. The enzyme is not active towards heparin or low molecular weight heparins. The lyase activity of Heparinase III had been previously characterised as "heparitinase I" by Silva, M.E. and Dietrich, C.P., (1974).

Particularly advantageous oligosaccharides according to the invention have at least some level of O- and/or N sulfation and may have levels of sulfation, or combinations thereof, as described below. In certain embodiments of the invention at least one O- and/or N-atom of a disaccharide "monomer" is sulphated. In further embodiments, at least one O- and/or N-atom of each disaccharide monomer is sulphated. Sulfation may occur at the C3 and/or C6 hydroxy groups and/or amino group of the glucosamine residue and/or at C2 and/or C3 of the uronic acid residue. In further embodiments sulfation occurs at C6 hydroxy and/or the amino group of the glucosamine residue and/or C2 hydroxy of the uronic acid residue. In some embodiments of the invention, at least 20-25% of the available O and N groups are

sulfonated. In further embodiments at least 30-40% are sulfonated. In yet other embodiments at least 40-50%, 50-60%, 60-70%, 70-80%, 80-90%, or 90-100% of O and N groups are sulfonated. In certain embodiments, the oligosaccharide has at least one of the glucosamine residues N-sulfated. Where appropriate, according to length, at least 2, 3, 4, 5, 6, 7 or 8 glucosamine residues are N-sulfated. In further embodiments of the invention all glucosamine residues are N-sulfated. In certain embodiments of the invention the oligosaccharide has at least one of the glucosamine residues O-sulfated at C3. Where appropriate, according to length, at least 2, 3, 4, 5, 6, 7 or 8 glucosamine residues are O-sulfated at C3. In further embodiments, no glucosamine residues are O-sulfated at C3. In certain embodiments the oligosaccharide has at least one of the glucosamine residues O-sulfated at C6. Where appropriate, according to length, at least 2, 3, 4, 5, 6, 7 or 8 glucosamine residues are O-sulfated at C6. In further embodiments of the invention all glucosamine residues are O-sulfated at C6. In certain embodiments of the invention, the oligosaccharide has at least one of the uronic acid residues O-sulfated at C2. Where appropriate, according to length, at least 2, 3, 4, 5, 6, 7 or 8 uronic residues are O-sulfated at C2. In further embodiments all uronic or glucuronic acid residues are O-sulfated at C2. In certain embodiments of the invention, the oligosaccharide has at least one of the uronic acid residues O-sulfated at C3. Where appropriate, according to length, at least 2, 3, 4, 5, 6, 7 or 8 uronic acid residues are O-sulfated at C3. In other embodiments, none of the uronic acid residues are O-sulfated at C3.

In particularly advantageous forms of the invention, the oligosaccharide is substantially fully N- and O-sulfated at N, and C6 of the glucosamine residues and at C2 of the uronic acid residues, i.e. at least 50%, 60%, 75%, 80%, 85%, 90%, 95% or 100% of the N and hydroxy C6 positions of glucosamine are sulfated and the C2 hydroxy of the uronic acid residues are sulfated.

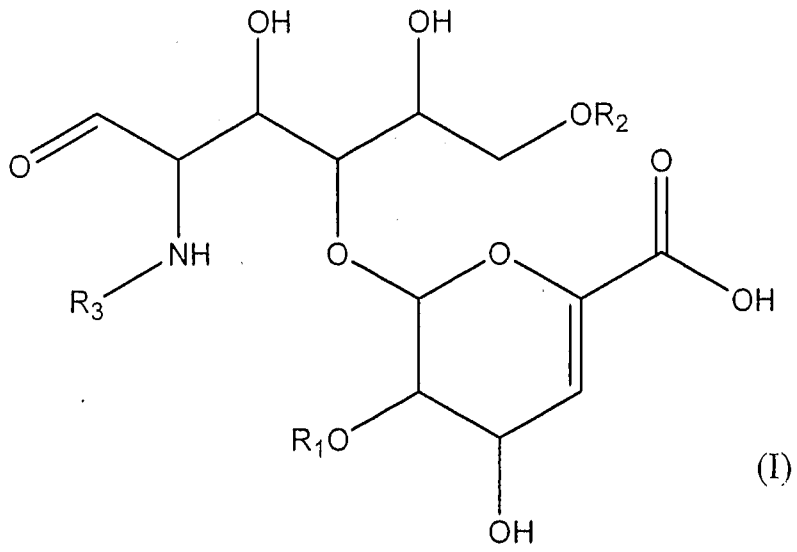
In certain embodiments of the invention the carboxyl group of the uronic acid residue remains intact, e.g. has not been reduced to the corresponding alcohol.

The fragment of heparin may have an inherent sulfation pattern or may be further

sulphated, using methods know in the art.

Heparin, or a derivative thereof may be sulfated prior to depolymerization or fractionation, or alternatively, the desired depolymerized or fractionated fraction can be subsequently sulfated.

An exemplary group of heparin fragments are the disaccharides of Formula (I), obtainable by heparinase digestion of heparin and/or heparan sulfate (McLean, M.W. *et al.* (1985)), as described and depicted below. These can also be obtained commercially (as their sodium salt) from various sources such as Sigma-Aldrich, Dextra Laboratories and Iduron Heparin Products. It will be appreciated that the depicted acyclic form of the glucosamine in Formula (I) is in equilibrium with the cyclic form and that the disaccharide could equally be depicted in its bicyclic form. In one embodiment of the invention, the disaccharides depicted by Formula (I) are α-linked (Sigma-Aldrich).



(I)

Heparin disaccharide I-A (ΔUA-2S-[1→ 4]-GlcNAc-6S)	$R_1 = SO_3H$,	$R_2 = SO_3H$,	$R_3 = Ac$
Heparin disaccharide I-H (ΔUA-2S-[1→ 4]-GlcN-6S)	$R_1 = SO_3H$,	$R_2 = SO_3H$,	$R_3 = H$
Heparin disaccharide I-S	$R_1 = SO_3H$,	$R_2 = SO_3H$,	$R_3 = SO_3H$

(ΔUA-2S-[1→ 4]-GlcNS-6S)			
Heparin disaccharide II-A	R_1 = H,	R_2 = SO_3H,	R_3 = Ac
(ΔUA-[1→ 4]-GlcNAc-6S)			
Heparin disaccharide II-H	R_1 = H,	R_2 = SO_3H,	R_3 = H
(ΔUA-[1→ 4]-GlcN-6S)			
Heparin disaccharide II-S	R_1 = H,	R_2 = SO_3H,	R_3 = SO_3H
(ΔUA-[1→ 4]-GlcNS-6S)			
Heparin disaccharide III-A	R_1 = SO_3H,	R_2 = H,	R_3 = Ac
(ΔUA-2S[1→ 4]-GlcNAc)			
Heparin disaccharide III-H	R_1 = SO_3H,	R_2 = H,	R_3 = H
(ΔUA-2S[1→ 4]-GlcN)			
Heparin disaccharide III-S	R_1 = SO_3H,	R_2 = H,	R_3 = SO_3H
(ΔUA-2S[1→ 4]-GlcNS)			
Heparin disaccharide IV-S	R_1 = H,	R_2 = H,	R_3 = SO_3H
(ΔUA-[1→ 4]-GlcNS			

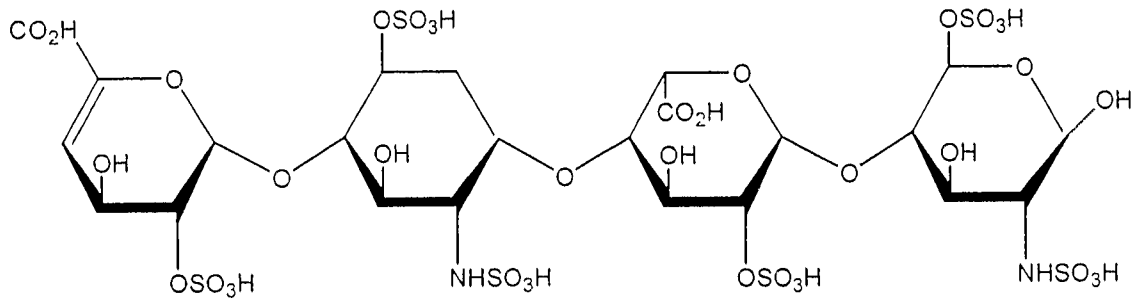
An exemplary disaccharide compound for use in the invention is α-ΔUA-2S-[1→ 4]-GlcNS-6S (Heparin disaccharide I-S). This compound is also variously referred to herein as 3S-HD or H9267.

For reference, non-sulfonated forms of heparin disaccharide are:

Heparin disaccharide IV-A	R_1 = H,	R_2 = H,	R_3 = Ac
(ΔUA-[1→ 4]-GlcNAc)			
Heparin disaccharide IV-H	R_1 = H,	R_2 = H,	R_3 = H
(ΔUA-[1→ 4]-GlcN)			

Further exemplary oligosaccharide compounds which may be suitable for use in the present invention include:

Heparin tetrasaccharide, HexA,2S-GlcNS,6S-(IdoA,2S-GlcNS,6S)



Heparin tetrasaccharide

Heparin hexasaccharide, HexA,2S-GlcNS,6S-(IdoA,2S-GlcNS,6S)$_2$

Heparin octasaccharide, HexA,2S-GlcNS,6S-(IdoA,2S-GlcNS,6S)$_3$

Heparin decasaccharide, HexA,2S-GlcNS,6S-(IdoA,2S-GlcNS,6S)$_4$

Heparin dodecasaccharide, HexA,2S-GlcNS,6S-(IdoA,2S-GlcNS,6S)$_5$

Heparin tetradecasaccharide, HexA,2S-GlcNS,6S-(IdoA,2S-GlcNS,6S)$_6$.

(HexA is the uronic acid residue at the reducing end which contains a C4-5 double bond as a result of endolytic action of bacterial heparinase. It should be noted that the main unit (Heparin tetrasaccharide, HexA,2S-GlcNS,6S-(IdoA,2S-GlcNS,6S) may contain some variation in sulfation from that depicted).

Further non-limiting examples include:

GlcA-aMan(6S)

IdoA-aMan(6S)

IdoA(2S)-aMan

GlcA(2S)-aMan

IdoA(2S)-aMan(6S)

GlcA(2S)-aMan(6S)

GlcA-GlcNAc(6S)-GlcA-aMan

IdoA(2S)-GlcNAc-GlcA-aMan

IdoA-GlcNAc(6S)-GlcA-aMan

IdoA(2S)-GlcNAc-GlcA-aMan(6S)

IdoA-GlcNAc(6S)-GlcA-aMan(6S), IdoA-GlcNAc(6S)-GlcA-aMan(3S)
IdoA-GlcNAc(6S)-GlcA-aMan(3,6-diS)
IdoA(2S)-GlcNAc(6S)-GlcA-aMan(6S)
IdoA(2S)-GlcNS(6S)-[IdoA(2S)-GlcNS(6S)]$_2$-GlcA/IdoA-aMan(6S)
IdoA(2S)-GlcNS(6S)-IdoA(2S)-GlcNS(6S)-GlcA/IdoA-aMan(6S)
IdoA(2S)-GlcNS(6S)-GlcA/IdoA-aMan(6S)
IdoA(2S)-GlcNS(6S)-[IdoA(2S)-GlcNS(6S)]$_3$-IdoA(2S)-aMan(6S)
IdoA(2S)-GlcNS(6S)-[IdoA(2S)-GlcNS(6S)]$_2$-IdoA(2S)-aMan(6S)
IdoA(2S)-GlcNS(6S)-IdoA(2S)-GlcNS(6S)-IdoA(2S)-aMan(6S)
IdoA(2S)-GlcNS(6S)-IdoA(2S)-aMan(6S)
(aMan = 2,5-anhydro-D-mannose).

Chemical means may also be used to obtain fragments of heparin and its derivatives. Chemical treatments may include: benzylation followed by alkaline treatment, peroxidative treatments, free radical attack and nitrous acid treatment.

Nitrous acid treatment of heparin cleaves a proportion of the α-D-N-sulfoglucosamine (GlcNS) residues , converting these to anhydromannose (see for example, Shively, J.E. and Conrad, H.E., (1976); Bienkowski, M.J., and Conrad, H.E., (1985); Pejler, G., *et al*, (1988) and Stringer, S.E., *et al* (2003)). Such depolymerization products, particularly those identified therein, are also contemplated by the invention.

It will be appreciated that the present invention also contemplates prodrugs of said agents. The term "prodrug" is used in its broadest sense and encompasses those derivatives that are converted *in vivo*, either enzymatically or hydrolytically, to the agents contemplated by the invention. Such derivatives would readily occur to those skilled in the art, and include, for example, compounds where a free or hydroxy or carboxylic group is converted into an ester, or where a free amino group is converted into an amide. Procedures for acylating the hydroxy groups of compounds, for example to prepare ester and amide prodrugs, are well known in the art and may include treatment of the compound with an appropriate carboxylic acid, anhydride or chloride in the presence of a suitable catalyst or base.

Carboxylic acids may be esterified by treatment with an alcohol in the presence of a suitable catalyst or base. Other conventional procedures for the selection and preparation of suitable prodrugs are known in the art and are described , for example, in WO 00/23419, *Design of Prodrugs*, Hans Bundgaard, Ed., Elsevier Science Publishers, 1985, and *The Organic Chemistry of Drug Desig and Drug Action*, Chapter 8, pp352-401, Academic press, Inc., 1992, the contents of which are incorporated herein by reference.

Suitable pharmaceutically acceptable salts of agents contemplated by the invention include, but are not limited to salts of pharmaceutically acceptable inorganic acids such as hydrochloric, sulphuric, phosphoric nitric, carbonic, boric, sulfamic, and hydrobromic acids, or salts of pharmaceutically acceptable organic acids such as acetic, propionic, butyric, tartaric, maleic, hydroxymaleic, fumaric, maleic, citric, lactic, mucic, gluconic, benzoic, succinic, oxalic, phenylacetic, methanesulphonic, toluenesulphonic, benezenesulphonic, salicyclic sulphanilic, aspartic, glutamic, edetic, stearic, palmitic, oleic, lauric, pantothenic, tannic, ascorbic and valeric acids. Base salts include, but are not limited to, those formed with pharmaceutically acceptable cations, such as sodium, potassium, lithium, calcium, magnesium, ammonium and alkylammonium. Basic nitrogen-containing groups may be quarternised with such agents as lower alkyl halide, such as methyl, ethyl, propyl, and butyl chlorides, bromides and iodides; dialkyl sulfates like dimethyl and diethyl sulfate; and others.

Accordingly, in the preceding embodiments of the present invention, the agent which inhibits the functional interactivity of heparin or heparan sulfate with said BACE1 zymogen pro-domain is preferably a fragment of heparin or a pharmaceutically acceptable salt thereof. In exemplary embodiments, the agent is a disaccharide of Formula (I), or pharmaceutically acceptable salt thereof. In other exemplary embodiments, the agent is a heparin tetrasaccharide. One particular example thereof is HexA, 2S-GLIcNS, 6S-(IdoA, 2S-GlcNS, 6S) (also referred to herein as 3S-HT).

As detailed above, in its preferred embodiment the present invention is directed to downregulating heparin or heparan sulfate induced BACE1 zymogen activity in the

context of minimising the β amyloid precursor protein cleavage event which leads to Aβ formation. Without limiting the present invention to any one theory or mode of action, Aβ is deposited in plaques, in particular in the brain, causing, *inter alia*, endothelial cell damage. It is therefore associated with cerebral vessel constriction, this correlating to one of the early clinical features of Alzheimer's disease – bilateral temporoparietal hypoperfusion. By minimising the generation of Aβ, amyloid plaque formation is necessarily reduced. Still further, whereas the most preferred outcome of treating Aβ amyloidosis is to entirely eliminate Aβ plaque formation, it is nevertheless extremely valuable if Aβ plaque formation can even be reduced somewhat, since there is a direct link between the extent of plaque formation and severity of clinical symptoms.

Accordingly, another aspect of the present invention is directed to a method of modulating the viability and/or functioning of Aβ amyloidogenic tissue in a subject, said method comprising administering to said subject an effective amount of an agent for a time and under conditions sufficient for said agent to antagonise the functional interactivity of heparan sulfate with the BACE1 zymogen pro-domain wherein antagonising said interaction inhibits or downregulates BACE1 zymogen mediated β amyloid precursor protein cleavage.

Preferably, said downregulated precursor protein cleavage corresponds to downregulated Aβ production.

Reference to "Aβ amyloidogenic tissue" should be understood as reference to any biological tissue which has undergone or otherwise become subject to Aβ derived amyloid deposition. For example, the subject tissue may be Aβ amyloidogenic encephalon tissue, as occurs in Alzheimer's Disease or some types of stroke.

Accordingly, the present invention preferably provides a method of modulating the viability and/or functioning of Aβ amyloidogenic encephalon tissue in a subject, said method comprising administering to said subject an effective amount of an agent for a time

and under conditions sufficient for said agent to antagonise the functional interactivity of heparan sulfate with the BACE1 zymogen pro-domain wherein antagonising said interaction inhibits or downregulates BACE1 zymogen mediated β amyloid precursor protein cleavage.

Preferably, said downregulated precursor protein cleavage corresponds to downregulated Aβ production.

Preferably, said antagonism is directed to the interaction of heparin or heparan sulfate with one or more basic amino acids of the BACE1 zymogen pro-domain and, more preferably, one or more of H24, R27, R31, R42 and/or R45.

In a preferred embodiment of this aspect, the agent which inhibits the functional interactivity of heparin or heparan sulfate with said BACE1 zymogen pro-domain is a fragment of heparin or a pharmaceutically acceptable salt thereof. In a particularly preferred embodiment, the agent is a disaccharide of Formula (I), or pharmaceutically acceptable salt thereof.

Reference to the "viability and/or functioning" should be understood as a reference to all forms of activity which the tissue performs including, but not limited to, all forms and aspects of cellular signalling, soluble mediator production, proliferation, differentiation, morphological and structural modulation (for example, such as occurs in skeletal muscle contraction) and maintenance of cellular viability. Most tissues comprise a heterogeneous population of cells. Accordingly, it should be understood that the subject viability and/or functioning may be modulated at the level of any one or more cells or sub-populations of cells within a given amyloidogenic tissue. To the extent that the subject tissue is preferably encephalon, reference to "encephalon functioning" should be understood as a reference to all forms of activity which the encephalon performs including, but not limited to, all forms of signalling which are either sent or received and interpreted by the encephalon, thought processes, memory function, and any other form of signalling or functioning which acts at the conscious, sub-conscious, physiological or anatomical level.

Preferably, the subject encephalon functional activity is cognitive functioning. By "cognitive functioning" is meant the operation of the human mind and includes, but is not limited to, functions such as thinking, memory, intelligence, rationalisation, emotion and behaviour. In this regard, reference to "encephalon" should be understood as a reference to the brain and includes, but is not limited to, the hindbrain (rhombencephalon), consisting of the medulla oblongata, pons Varolii and cerebellum and the midbrain (mesencephalon) and the forebrain (prosencephalon) which is subdivided into the cerebrum and the diencephalon (including the thalamus and hypothalamus).

Accordingly, another aspect of the present invention is directed to a method of upregulating Aβ amyloidogenic encephalon cognitive functioning in a subject, said method comprising administering to said subject an effective amount of an agent for a time and under conditions sufficient for said agent to antagonise the functional interactivity of heparan sulfate with the BACE1 zymogen pro-domain wherein antagonising said interaction inhibits or downregulates BACE1 zymogen mediated β amyloid precursor protein cleavage.

Preferably, said downregulated precursor protein cleavage corresponds to downregulated Aβ production.

Preferably, said antagonism is directed to the interaction of heparin or heparan sulfate with one or more basic amino acids of the BACE1 zymogen pro-domain and, more preferably, one or more of H24, R27, R31, R42 and/or R45.

In a preferred embodiment of this aspect, the agent which inhibits the functional interactivity of heparin or heparan sulfate with said BACE1 zymogen pro-domain is a fragment of heparin or a pharmaceutically acceptable salt thereof. In a particularly preferred embodiment, the agent is a disaccharide of Formula (I), or pharmaceutically acceptable salt thereof.

Reference to "Aβ", "amyloid" and "Aβ amyloid", in terms of the deposition of amyloid, should be understood as a reference to all classes and forms of Aβ molecules which are derived from cleavage of the β amyloid precursor protein including, for example, any isoforms which arise from alternative splicing of Aβ mRNA or allelic or polymorphic variants of Aβ or precursor forms of Aβ. Without limiting the present invention in any way, amyloid precursor protein comprises a core of amyloid fibrils surrounded by dystrophic neurites. The principal component of the amyloid fibrils is B/A4, being a peptide derived from the larger amyloid precursor protein. The A4 protein is an integral membrane glycoprotein of the brain. Aβ can vary in length from 39 to 43 amino-acid residues, with the most common forms containing 40- or 42-amino acids (Aβ40 or Aβ42) (Small *et al.*, 1999, *J Neurochem* 73, 443-449). Aβ is relatively hydrophobic and shows a propensity to aggregate into oligomers and higher molecular weight amyloid fibrils. Indeed, there is now considerable evidence that the accumulation of the oligomeric forms of Aβ causes the neurotoxicity that underlies the neurodegeneration in AD (Small *et al.*, 2001, *Nature Rev. Neurosci.* 2, 595-598.). However, the mechanism by which Aβ generates this neurotoxicity is poorly understood. Aβ is produced by proteolytic cleavage of a much larger (110-130 kDa) precursor known as the β-amyloid precursor protein (APP). APP has features of an integral type I transmembrane glycoprotein (Fig. 10).

The Aβ domain comprises part of the ectodomain of the protein and extends into the transmembrane domain. APP can be cleaved within the Aβ domain by α-secretase. This cleavage destroys the intact Aβ sequence and is therefore considered to be a non-amyloidogenic cleavage pathway (Small *et al.*, 1999, *supra*; Nunan *et al.*, 2000, *FEBS Lett* 483, 6-10). Alternatively, APP can be cleaved on the N-terminal side of the Aβ domain by BACE1 (an acronym for β-site APP cleaving enzyme-1). This cleavage generates a C-terminally truncated form of APP (sAPPβ) and a C-terminal fragment known as C-99, which is subsequently cleaved by γ-secretase, a multiprotein complex comprising presenilin-1 or 2, nicastrin, Aph-1 and Pen-2 to generate Aβ (Nunan *et al.*, 2000, *supra*). Accordingly, reference to "Aβ" includes reference to Aβ40, Aβ42 or the 38, 39 or 43

amino acid length forms of Aβ.

The term "subject" as used herein includes humans, primates, livestock animals (eg. sheep, pigs, cattle, horses, donkeys), laboratory test animals (eg. mice, rabbits, rats, guinea pigs), companion animals (eg. dogs, cats), captive wild animals (eg. foxes, kangaroos, deer), aves or reptiles. Preferably, the mammal is human or a laboratory test animal Even more preferably, the mammal is a human.

An "effective amount" means an amount necessary at least partly to attain the desired response, or to delay the onset or inhibit progression or halt altogether, the onset or progression of a particular condition being treated. The amount varies depending upon the health and physical condition of the individual to be treated, the taxonomic group of individual to be treated, the degree of protection desired, the formulation of the composition, the assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

Without limiting the present invention to any one theory or mode of action, and to the extent that the present invention is preferably directed to a method of treating Aβ amyloidogenic encephalon tissue, it is thought that the method of the present invention downregulates Aβ generation, thereby effectively downregulating plaque formation. The development of the method of the present invention has facilitated the design and application of therapeutic and prophylactic protocols for treating conditions characterised by encephalon functioning which has been impaired directly or indirectly due to amyloidosis.

Reference to the treatment and/or prophylaxis of the subject condition should be understood as a reference to the treatment of any disease or other condition, the symptoms, cause or side effects of which include aberrant, unwanted or otherwise undesirable Aβ amyloidosis, irrespective of whether symptoms in this regard are evident. This includes, for example, conditions which occur as a side effect of a treatment regime for an unrelated

disease condition. The subject disease condition may be congenital or acquired and may be in an acute or chronic phase.

Preferably, the subject condition is Alzheimer's Disease, Down's Syndrome, cerebral haemorrhage with Aβ amyloidosis, Aβ amyloidosis associated with chronic inflammation and β amyloidosis associated with senile cardiac amyloid.

The method of the present invention preferably facilitates the subject condition being reduced, retarded or otherwise inhibited. Reference to "reduced, retarded or otherwise inhibited" should be understood as a reference to inducing or facilitating the partial or complete inhibition of any one or more causes or symptoms of the subject condition. In this regard, it should be understood that conditions such as Alzheimer's Disease are extremely complex comprising numerous physiological events which often occur simultaneously. In terms of the object of the subject method of treatment and/or prophylaxis, it should be understood that the present invention contemplates both relieving any one or more symptoms of the subject condition (for example, improving one or more cognitive functions) or facilitating retardation or cessation of the cause of the disease condition (for example, reducing devascularisation events which are related to ongoing Aβ deposition thereby minimising any further encephalon damage).

Accordingly, another aspect of the present invention is directed to a method for the treatment and/or prophylaxis of a condition characterised by impaired tissue viability and/or functioning, which impaired tissue viability and/or functioning is directly or indirectly induced by Aβ amyloidosis, said method comprising administering to said subject an effective amount of an agent for a time and under conditions sufficient for said agent to antagonise the functional interactivity of heparan sulfate with the BACE1 zymogen pro-domain wherein antagonising said interaction inhibits or downregulates BACE1 zymogen mediated β amyloid precursor protein cleavage.

Preferably, said downregulated precursor protein cleavage corresponds to downregulated Aβ production.

More preferably, said antagonism is directed to the interaction of heparan sulfate with one or more basic amino acids of the BACE1 zymogen pro-domain and, still more preferably, one or more of H24, R27, R31, R42 and/or R45.

In a preferred embodiment of this aspect, the agent which inhibits the functional interactivity of heparin with said BACE1 zymogen pro-domain is a fragment of heparin or a pharmaceutically acceptable salt thereof. In a particularly preferred embodiment, the agent is a disaccharide of Formula (I), or pharmaceutically acceptable salt thereof.

Accordingly, the present invention preferably provides a method for the treatment and/or prophylaxis of a condition characterised by encephalon Aβ deposition, said method comprising administering to said subject an effective amount of an agent for a time and under conditions sufficient for said agent to antagonise the functional interactivity of heparan sulfate with the BACE1 zymogen pro-domain wherein antagonising said interaction inhibits or downregulates BACE1 zymogen mediated β amyloid precursor protein cleavage.

Preferably, said downregulated precursor protein cleavage corresponds to downregulated Aβ production.

More preferably, said antagonism is directed to the interaction of heparan sulfate with one or more basic amino acids of the BACE1 zymogen pro-domain and, still more preferably, one or more of H24, R27, R31, R42 and/or R45.

In a preferred embodiment of this aspect, the agent which inhibits the functional interactivity of heparan sulfate with said BACE1 zymogen pro-domain is a fragment, or pharmaceutically acceptable salt thereof, of heparin or a derivative thereof. In a particularly preferred embodiment, the agent is a disaccharide of Formula (I), or pharmaceutically acceptable salt thereof.

Preferably, the subject condition is Alzheimer's Disease, Down's Syndrome, cerebral haemorrhage with Aβ amyloidosis, Aβ amyloidosis associated with chronic inflammation and β amyloidosis associated with senile cardiac amyloid.

Still more preferably, said tissue is encephalon and said amyloidosis is induced by Aβ deposition on the encephalon. Most preferably said condition is Alzheimer's Disease or Down's Syndrome.

Still more preferably the present invention provides a method for the treatment and/or prophylaxis of Alzheimer's disease in a subject, said method comprising administering to said subject an effective amount of an agent for a time and under conditions sufficient for said agent to antagonise the functional interactivity of heparan sulfate with the BACE1 zymogen pro-domain wherein antagonising said interaction inhibits or downregulates BACE1 zymogen mediated β amyloid precursor protein cleavage.

Preferably, said downregulated precursor protein cleavage corresponds to downregulated Aβ production.

Preferably, said antagonism is directed to the interaction of heparan sulfate with one or more basic amino acids of the BACE1 zymogen pro-domain and, more preferably, one or more of H24, R27, R31, R42 and/or R45.

In a preferred embodiment of this aspect, the agent which inhibits the functional interactivity of heparin with said BACE1 zymogen pro-domain is a fragment of heparin or a pharmaceutically acceptable salt thereof. In a particularly preferred embodiment, the agent is a disaccharide of Formula (I), or pharmaceutically acceptable salt thereof.

Administration of the agent (herein referred to as the "modulatory agent"), in the form of a pharmaceutical composition, may be performed by any convenient means. The modulatory agent of the pharmaceutical composition is contemplated to exhibit therapeutic activity when administered in an amount which depends on the particular case. The

variation depends, for example, on the human or animal and the form of modulatory agent chosen. A broad range of doses may be applicable. Considering a patient, for example, from about 0.1 mg to about 1 mg of modulatory agent may be administered per kilogram of body weight per day. Dosage regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily, weekly, monthly or other suitable time intervals or the dose may be proportionally reduced as indicated by the exigencies of the situation. The modulatory agent may be administered in a convenient manner such as by the oral, intravenous (where water soluble), intraperitoneal, intramuscular, subcutaneous, intradermal or suppository routes or implanting (e.g. using slow release molecules). Preferably the agent is administered orally and dosages of 1-10 grams per day are envisioned. More particularly the dosage is 2-6 grams per day. The modulatory agent may be administered in the form of pharmaceutically acceptable nontoxic salts, such as acid addition salts or metal complexes, e.g. with zinc, iron or the like (which are considered as salts for purposes of this application). Illustrative of such acid addition salts are hydrochloride, hydrobromide, sulphate, phosphate, maleate, acetate, citrate, benzoate, succinate, malate, ascorbate, tartrate and the like. If the active ingredient is to be administered in tablet form, the tablet may contain a binder such as tragacanth, corn starch or gelatin; a disintegrating agent, such as alginic acid; and a lubricant, such as magnesium stearate.

Routes of administration include, but are not limited to, respiratorally, intratracheally, nasopharyngeally, intravenously, intraperitoneally, subcutaneously, intracranially, intradermally, intramuscularly, intraoccularly, intrathecally, intracereberally, intranasally, infusion, orally, rectally, *via* IV drip patch and implant. Preferably, said route of administration is oral.

In yet another aspect, the present invention relates to the use of a modulatory agent in the manufacture of a medicament for the prophylactic and/or therapeutic treatment of a condition characterised by impaired tissue viability and/or functioning, which impaired viability and/or functioning is directly or indirectly induced by amyloidosis, wherein said

modulatory agent antagonises the functional interactivity of heparan sulfate with the BACE1 zymogen pro-domain.

In a preferred embodiment of this aspect, the agent which inhibits the functional interactivity of heparin with said BACE1 zymogen pro-domain is a fragment of heparin or a pharmaceutically acceptable salt thereof. In a particularly preferred embodiment, the agent is a disaccharide of Formula (I), or pharmaceutically acceptable salt thereof.

More preferably, the subject condition is Alzheimer's Disease, Down's Syndrome, cerebral haemorrhage with Aβ amyloidosis, Aβ amyloidosis associated with chronic inflammation and β amyloidosis associated with senile cardiac amyloid.

Most preferably, said condition is Alzheimer's Disease.

In yet another aspect, the present invention relates to the use of a modulatory agent in the manufacture of a medicament for the prophylactic and/or therapeutic treatment of a condition characterised by encephalon Aβ deposition, wherein said modulatory agent antagonises the functional interactivity of heparan sulfate with the BACE1 zymogen pro-domain.

Preferably, said functional interactivity antagonism downregulates β amyloid precursor protein cleavage.

Preferably, said downregulated precursor protein cleavage corresponds to downregulated Aβ production.

Preferably, said antagonism is directed to the interaction of heparin or heparan sulfate with one or more basic amino acids of the BACE1 zymogen pro-domain and, more preferably, one or more of H24, R27, R31, R42 and/or R45.

In a preferred embodiment of this aspect, the agent which inhibits the functional

interactivity of heparin with said BACE1 zymogen pro-domain is a fragment of heparin or a pharmaceutically acceptable salt thereof. In a particularly preferred embodiment, the agent is a disaccharide of Formula (I), or pharmaceutically acceptable salt thereof.

In a related aspect of the present invention, the mammal undergoing treatment may be a human or animal in need of therapeutic or prophylactic treatment. Preferably, said mammal is a human.

In accordance with these methods, the modulatory agent defined in accordance with the present invention may be coadministered with one or more other compounds or molecules. By "coadministered" is meant simultaneous administration in the same formulation or in two different formulations via the same or different routes or sequential administration by the same or different routes. By "sequential" administration is meant a time difference of from seconds, minutes, hours or days between the administration of the two types of molecules. These molecules may be administered in any order.

The method of the present invention may also be combined with currently known methods of treatment such as, in relation to Alzheimer's Disease for example, treating associated non-cognitive problems, treating patients with cholinesterase inhibitors (donepezil, rivastigmine and galantamine), which provide symptomatic treatments and have been shown to improve cognitive functioning, or treating patients with Aricept.

In yet another aspect the present invention relates to a pharmaceutical composition comprising modulatory agent as hereinbefore defined and one or more pharmaceutically acceptable carriers and/or diluents. Said pharmaceutical composition may additionally comprise molecules with which it is to be co-administered. These agents are referred to as the active ingredients.

In a preferred embodiment of this aspect, the agent which inhibits the functional interactivity of heparin with said BACE1 zymogen pro-domain is a fragment of heparin or a pharmaceutically acceptable salt thereof. In a particularly preferred embodiment, the

agent is a disaccharide of Formula (I), or pharmaceutically acceptable salt thereof.

Although the method of the present invention is preferably achieved via the oral administration of the subject agent, it should be understood that the present invention is not limited to this method of administration and may encompass any other suitable method of administration. In this regard, the pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion or may be in the form of a cream or other form suitable for topical application. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of superfactants. The preventions of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilisation. Generally, dispersions are prepared by incorporating the various sterilised active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying

technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

When the active ingredients are suitably protected they may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of active compound in such therapeutically useful compositions in such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 0.1 μg and 3000 mg of active compound.

The tablets, troches, pills, capsules and the like may also contain the components as listed hereafter: a binder such as gum, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound(s) may be incorporated into sustained-release preparations and formulations.

Yet another aspect of the present invention relates to modulatory agents, as hereinbefore defined, when used in the method of the present invention.

The present invention is further described by reference to the following non-limiting examples.

EXAMPLE 1

HEPARIN ACTIVATES β-SECRETASE (BACE1) OF ALZHEIMER'S DISEASE AND INCREASES AUTOCATALYSIS OF THE ENZYME

Materials and Methods

Materials

rBACE1, amino acid residues 1-460 with or without a C-terminal His-tag was obtained from R&D Systems (#931-AS, Bioscientific, Gymea, Australia) and Invitrogen (#P2947, Mount Waverley, VIC, Australia), respectively. The substrate peptide MCA-SEVNLDAEFRK(DNP)RR-NH_2 (SEQ ID NO:2) was purchased from MP Biomedicals (Seven Hills, NSW, Australia) and microplates (OptiPlate-96 F) from PerkinElmer Life Sciences (Boston, MA, USA). Bovine serum albumin (BSA, fraction V), Coomassie blue R-250, heparin from porcine intestinal mucosa (H3393), human recombinant apolipoprotein E4 (apoE4), polyclonal N-terminal anti-BACE1 antibody (immunogen peptide comprising of amino acids 46-62 of human BACE1; EE-17) and protein A-Sepharose 4B Fast Flow were all obtained from Sigma-Aldrich (Sydney, Australia). HiTrap Heparin HP columns, anti-rabbit IgG HRP and ECL reagent were from Amersham Biosciences (Castle Hill, Australia) and Centricon YM-10 and Microcon YM-30 concentrators, Immobilon-P membrane (0.45 μm) and C18 ZipTips from Millipore (North Ryde, Australia). A peptide homologous to the pro-domain of BACE1 (TQHGIRLPLRSGLGGAPLGLRLPR-NH_2) (SEQ ID NO:3) was synthesised by Auspep (Parkville, Australia). Polyclonal anti-proBACE1 antibody (PRB622C, Covance) was purchased from Chemicon (Boronia, Australia) and the Penta-His HRP conjugate kit from Qiagen (Doncaster, Australia). Bio-Rad Silver Stain and Sequi-Blot PVDF membrane were obtained from Bio-Rad Laboratories (Regents Park, Australia) and syringe filters from Pall Corporation (Cheltenham, Australia). The Brownlee Spheri-5 RP-18 column was purchased from Applied Biosystems (Scoresby, Australia). Heparin disaccharide I-S sodium salt (H9267) was purchased from Sigma-Aldrich (Australia).

BACE1 activity assay

BACE1 activity was measured using a quenched- fluorescence assay with a peptide substrate homologous to the Swedish APP mutant β-cleavage site (Yan *et al.*, 1999, *supra*; Holsinger *et al.*, 2004, *Ann. Neurol. 55*, 898-899). Assays were performed in a buffer of 0.1 M sodium acetate pH 4, 5 % dimethylsulfoxide (DMSO) using 0.8 μg rBACE1 (unless otherwise specified) and 1-20 μM substrate peptide in a total volume of 100 μL in black 96-well plates. Heparin was dissolved in water and sterile filtered with a 0.45 μm syringe filter. The increase in fluorescence intensity (F) produced by substrate hydrolysis was monitored at 37 °C on a fluorescence microplate reader (FLUOstar, BMG Lab Technologies, Offenburg, Germany) with excitation and emission wavelengths of 320 and 405 nm, respectively. Enzyme activity was calculated from the change in F per minute (ΔF/min) during the initial linear phase of the reaction and plotted against increasing substrate concentrations. The data were fitted to the Michaelis-Menten equation by the method of least squares using GraphPad Prism software (version 4.00) to calculate V_{max} and K_M.

Gel electrophoresis and western blotting

Samples (10 to 25 μL) were mixed with 2x SDS sample buffer and heated to 95 °C for 5 min prior to gel loading. The proteins were separated by 10 % SDS-PAGE (Laemmli 1970, *Nature 227*, 680-685) and either silver stained using BioRad Silver Stain reagent, or electroblotted onto an Immobilon-P membrane for one hour at 350 mA. For immunoblot analysis with the polyclonal antibodies EE-17 and anti-proBACE1, the membrane was blocked in 5 % skim milk powder in PBS with 0.05 % Tween 20, pH 7.4, and incubated with the antibodies diluted to 1:2000 and 1:1000, respectively, in blocking solution. HRP-conjugated secondary antibody was used for chemiluminescent detection by ECL. For detection of the His-tag, a Penta-His HRP conjugate kit was used according to the manufacturer's instructions (Qiagen, Doncaster, Australia) and immunoblots were developed using ECL. An image of the signals obtained on X-ray film was acquired and band intensities were measured using AlphaImager with AlphaEase Software (Quantum

Scientific, Victoria, Australia).

N-terminal amino acid sequencing

Samples of rBACE1 (6 -7.5 μg) were separated by 10 % SDS-PAGE and electroblotted onto a Sequi-Blot PVDF membrane using 10 mM CAPS buffer with 10 % methanol (Matsudaira, 1987, *J. Biol. Chem. 262*, 10035-10038). The membrane was stained with Coomassie Blue R-250 (0.1 % in 40 % methanol, 1 % acetic acid) and then destained in 50% methanol until the background was light blue. The membrane was then washed in water, air dried, and the band of interest excised. Proteins on the excised membrane were subjected to N-terminal amino acid sequencing by an automated Edman degradation procedure using the Procise Protein Sequencing System (Applied Biosystems, Scoresby, Australia).

Heparin affinity chromatography

For heparin affinity chromatography, 15 μg of rBACE1 (100 μg/mL in distilled water) or 250 μg of peptide (1 mM in dimethyl sulfoxide, DMSO) were diluted to 1 mL with 10 mM sodium acetate buffer pH 5 (acetate buffer) and then applied to a 1 mL HiTrap Heparin HP column pre-equilibrated with acetate buffer. Unbound material was eluted with 2x3 mL of acetate buffer and then bound material was eluted with 1x3 mL washes of 0.15 M, 0.5 M and 1.2 M NaCl in acetate buffer at a flow rate of 1 mL/min. For rBACE1, fractions of 3 mL were collected and then concentrated (11-fold) and the buffer exchanged to 0.1 M sodium acetate pH 4 using Centricon YM-10.

Reversed-phase high performance liquid chromatography (RP-HPLC)

Elution of a synthetic peptide homologous to the pro-domain of BACE1 from the heparin column was monitored by RP-HPLC. A portion (0.9 mL) of the heparin column wash fractions or a standard amount of the peptide (90 μg) was applied to a 4.6 x 220 mm Brownlee Spheri-5 RP18 column (5 μm bead size, 80 Å pore size) using an Agilent

Technologies 1100 Series solvent delivery system (Forest Hill, Australia). Samples were filtered through a 0.45 μm syringe filter before injection onto the column. After an initial wash for 5 min with solvent A (0.1 % trifluoroacetic acid, TFA, in water), the peptide was eluted with a linear gradient of 0-70 % solvent B (0.1 % TFA in 90 % acetonitrile/water) over 35 min at a flow rate of 1 mL/min. The elution of the peptide from the column was monitored by measuring the absorbance at a wavelength of 218 nm.

Immunoprecipitation

Immunoprecipitation was performed with the anti-proBACE1 antibody (1 μl) and protein A-Sepharose (30 μl). Briefly, protein A beads were pre-loaded with the antibody in PBS and the mixture was rotated for 1 h at room temperature. The beads were centrifuged and washed in PBS, after which 2 μg rBACE1 (R&D Systems) in PBS (500 μl) was added. The mixture was rotated overnight at 4 °C. The beads were centrifuged and the supernatant fraction collected and concentrated (7.5-fold) using Microcon YM-30. A sample (20 %) of the concentrated supernatant was subjected to western blot analysis and enzyme activity was measured in the absence or presence of heparin (1 μg/mL) in the remaining part of the fraction.

Analysis by mass spectrometry (MS)

MALDI-TOF MS analysis was performed with a 4700 Proteomics Analyser (Applied Biosystems, Scoresby, Australia). Prior to loading on the sample plate, the samples were desalted using C18 ZipTips according to the manufacturer's instructions. The instrument was operated in positive polarity in a linear mode or reflector mode using alpha-cyano-4-hydroxycinnamic acid matrix (Agilent Technologies, Forest Hill, Australia) and calibrated against 6 peptides of the Applied Biosystems supplied 4700 mix. Matrix (0.5 μL) was spotted on the sample plate and allowed to air dry. Samples (0.5 μL) were subsequently spotted on dried matrix and allowed to air dry. Data were collected and processed with the instrument manufacturer's 4000 series Explorer software (version 3). Tandem MS analysis (MSMS) was performed by precursor selection of the mass of the peptide of interest and

the masses of the resulting MSMS spectrum were compared with the theoretical fragment masses of the expected sequence generated by the ion fragmentation calculator contained as a part of the 4000 series Explorer software.

Results

Effect of heparin on rBACE1 activity

Initially, the effect of various concentrations of heparin on BACE1 activity was examined. Similar to a previous report (Scholefield *et al.*, 2003, *J. Cell Biol. 163*, 97-107), heparin was found to inhibit the activity of rBACE1. However, this inhibition occurred only at relatively high concentrations of heparin (10 or 100 μg/mL) (Fig. 1 A). At a lower concentration of heparin (1 μg/mL), the enzyme activity was increased (Fig. 1 A). To examine whether this increase in activity was due to an effect on the V_{max} or K_M of the enzyme reaction, the ability of 1 μg/mL heparin to stimulate activity over a range of substrate concentrations (1 to 30 μM) was examined. Results showed that the V_{max} was increased to almost 200 % of the control value in the presence of 1 μg/mL heparin and the K_M was decreased from approximately 11.4 μM in the absence of heparin to 6.5 μM in the presence of 1 μg/mL heparin (Fig. 1 B).

The specificity of the heparin-induced stimulation of BACE1 was investigated by testing whether or not a heparin-binding protein, apolipoprotein E (apoE) (Dong *et al.*, 2001, *Biochemistry 40*, 2826-2834), could compete with rBACE1 for binding to heparin and thereby inhibit the stimulation. Consistent with this idea, the stimulatory effect of heparin on enzyme activity was inhibited by apoE (Fig. 1 C). ApoE had no effect on basal enzyme activity in the absence of heparin, indicating that the ability of apoE to block the heparin-induced stimulation was due to an interaction between apoE and heparin and not between apoE and BACE1.

The rBACE1 used in the experiments described above was obtained from R&D Systems and contained a His-tag at the C-terminus. To exclude the possibility that the stimulatory

effect of heparin might be due to an interaction between heparin and the His-tag, the ability of heparin to stimulate rBACE1 activity using an enzyme preparation from Invitrogen, which lacked a His-tag was examined. The activity of the Invitrogen rBACE1 was increased by 54 % in the presence of 1 μg/mL heparin when using 5 μM substrate peptide (n=3, $P<0.005$, Student's *t*-test). Thus, heparin was able to stimulate rBACE1 activity independently of the His-tag. In all further experiments, the His-tagged enzyme was used.

Analysis of the time course of activation showed that 1 μg/mL heparin had a dual effect on rBACE1 (Fig. 2). In an initial phase, heparin increased enzyme activity. However, the increased activity was followed by a second phase in which enzyme activity was lost. The effect of heparin was dependent on the concentration of the substrate peptide. At a very low substrate concentration (0.5 μM), the inhibition of substrate peptide cleavage occurred very fast (Fig. 2 A). When the concentration of substrate was increased to 1 μM, significant stimulation of enzyme activity was seen during the first 15 min of incubation (Fig. 2 B). After the phase of activation in the presence of 1 μM substrate, there was a lower rate of substrate hydrolysis as compared to the control incubations lacking heparin. The observed loss of enzyme activity was not due to consumption of the substrate. In the incubations with heparin, the rate of substrate cleavage at later time points (between 90 and 165 min) was lower than in control incubations both at 0.5 μM and 1 μM substrate (n=2-6, $P<0.0001$, Student's *t*-test). This showed that the inhibitory phase was due to a decline in enzyme activity. At higher substrate concentrations (5 or 20 μM), the heparin-induced stimulation phase was more prolonged than at the lower concentrations, and the subsequent phase of inhibition was delayed (Fig. 1 A and 2). Thus, the length of the stimulatory phase induced by heparin was dependent on the concentration of substrate peptide.

Effect of heparin on autocatalytic cleavage of rBACE1

BACE1 is known to undergo autocatalytic cleavage in the protease domain (Lin *et al.*, 2000, *supra*). Because the rate of autocatalysis would be lower at higher substrate concentrations, it was hypothesised that the delayed appearance of the inhibitory phase at higher substrate concentrations may have been due to a lower rate of autocatalysis at these

higher concentrations. Therefore, whether or not heparin could promote autocatalytic cleavage of rBACE1 was investigated. rBACE1 was incubated in the absence or presence of heparin and aliquots taken at different time points were analysed on silver-stained SDS-polyacrylamide gels and by western blotting. The results showed that when using a low molar ratio of heparin to rBACE1 (1 μg/mL heparin: 8 μg/mL rBACE1), which stimulates enzyme activity, autocatalytic cleavage was increased. In contrast, little autocatalysis was observed at a concentration of heparin (100 μg/mL) that inhibited enzyme activity.

Two major bands (M_r = 70-73 kDa and 65-68 kDa) of rBACE1 were observed on silver-stained gels (control, Fig. 3 A). Western blotting using the antibody EE-17 directed against amino acids 46-62 of BACE1 or an antibody directed against the C-terminal His-tag also revealed two immunoreactive bands of rBACE1, a major band at 70-73 kDa and a minor at 65-68 kDa (control, Fig. 3 C and E). The difference in molecular mass between the two bands (5 kDa) was greater than would have been expected from cleavage of the 2.5 kDa pro-domain sequence alone. The protein migrating at 70-73 kDa was recognised by an antibody directed against the pro-sequence of BACE1 (control, Fig. 3 C and E). A small amount of pro-enzyme immunoreactivity was also detected in association with the 65-68 kDa band. N-terminal amino acid sequencing showed that the upper 70-73 kDa band predominantly contained the zymogen (pro-rBACE1) beginning at threonine-22, but it also contained a small amount of mature enzyme beginning at glutamate-46. However, because the 65-68 kDa band also contained a small amount of pro-rBACE1, this indicated that proteolytic cleavage of the pro-domain was not solely responsible for the difference in molecular mass. Instead, it is likely that differential glycosylation accounted for some of this difference. Therefore, the preparation of rBACE1 consisted mainly of the zymogen, which preferentially migrated at an apparent molecular mass of 70-73 kDa.

After incubation with heparin (1 μg/mL) for 15 min, the amount of the 70-73 kDa form decreased and several fragments of lower relative molecular mass (40 to 62 kDa) were generated (Fig. 3 A). These fragments were recognised by an antibody directed against the C-terminal His-tag, identifying them as C-terminal fragments (Fig. 3 B). Western blot analysis using the anti-proBACE1 antibody showed that the 70-73 kDa pro-enzyme was

decreased after 5 min of incubation with heparin (1 μg/mL) (Fig. 3 C). The rate of loss of the zymogen after incubation with 1 μg/mL heparin was much higher at pH 4 (Fig. 3 C) than at pH 7 (Fig. 3 D), confirming that the breakdown was due to autocatalytic cleavage, as BACE1 activity is much higher at acidic pH. In the presence of a high concentration of heparin (100 μg/mL), there was little autocatalytic cleavage both at acidic and neutral pH (Fig. 3 C and D).

Effect of substrate peptide on heparin-stimulated autocatalytic cleavage of rBACE1

As previous experiments showed that high concentrations of substrate peptide delayed the secondary inhibitory phase of enzyme activity, whether or not the substrate peptide also affected the rate of autocatalysis was examined. Heparin-induced autocatalytic cleavage was found to proceed at a slower rate in the presence of substrate peptide (5 μM) than in its absence (Fig. 3 E). This was also demonstrated by quantification of the amount of pro-domain and total (i.e., C-terminal His and EE-17) BACE1 immunoreactivity associated with the 70-73 kDa band after western blotting (Fig. 3 F and G). Because higher concentrations of substrate peptide both decreased the rate of autocatalysis and prolonged the heparin-induced stimulation, this suggested that the later inhibitory phase that followed after the activation was due to autocatalytic cleavage of the enzyme.

Analysis of the pro-rBACE1 immunoreactivity indicated that it was more rapidly lost in the presence of heparin (1 μg/mL) than total rBACE1 immunoreactivity (Fig. 3 F and G). This suggested that N-terminal pro-sequence cleavage might occur at a faster rate than other autocatalytic cleavages, thus providing a mechanism for the increased activity of the enzyme. Alternatively, the zymogen may be more sensitive to autocatalytic degradation than the mature enzyme. To distinguish between these possibilities, the 70-73 kDa form of rBACE1 was subjected to N-terminal amino acid sequencing after activation of rBACE1 for 5 min with heparin. The result showed that after incubation with heparin, the enzyme was predominantly in the zymogen form starting at threonine-22, indicating that heparin did not increase production of the mature form. Therefore, the observed loss of pro-rBACE1 immunoreactivity most likely indicated that the zymogen was more susceptible to

autodegradation than the mature form. In addition, proteolytic removal of the pro-domain appeared not to be necessary for the activation by heparin.

Heparin affinity chromatography of rBACE1

Heparin is known to bind to clusters of positively charged residues on the surface of proteins (Hileman *et al.*, 1998, *Bioessays 20*, 156-167). Analysis of the tertiary folding of mature BACE1 did not reveal any obvious domain that could form a high-affinity binding site for heparin. However, as the main preparation of rBACE1 used in this study contained a mixture of both the pro- and the mature (pro-sequence cleaved) enzyme, and as the pro-sequence (residues 22-45) contains 5 positively charged residues, it was hypothesised that the heparin-induced stimulation of BACE1 was caused by binding of heparin to the pro-sequence.

To analyse the binding of BACE1 to heparin further, rBACE1 was applied to a heparin column, which was then eluted with buffers of increasing salt concentration. Western blot analysis of the salt washes showed that pro-rBACE1 bound strongly to heparin as it eluted primarily in the 1.2 M NaCl fraction (Fig. 4 A). Next, the enzyme activity in the fractions collected from the heparin column was analysed. The enzyme eluting in the 1.2 M NaCl fraction was strongly activated by heparin (1 μg/mL) (Fig. 4 B). As the pro-domain immunoreactivity was recovered predominantly in this fraction, this result supported the view that the zymogen was the target for the heparin-induced stimulation.

Heparin affinity chromatography of a synthetic BACE1 pro-peptide

To assess the heparin-binding capacity of the pro-domain, whether or not a synthetic peptide homologous to the pro-domain could bind to the heparin column was examined. A pro-domain peptide was applied to the column, eluted with a step-wise salt gradient and the collected fractions were assayed for the peptide by RP-HPLC. The peptide bound to the column and was recovered exclusively in the 0.5 M NaCl fraction (Fig. 5), supporting the view that the BACE1 pro-peptide is able to interact with heparin.

Effect of heparin on mature rBACE1

Data indicated that heparin-induced activation of enzyme activity may be mediated by an interaction of heparin with the pro-domain of the enzyme. This would mean that the mature (pro-sequence cleaved) enzyme should be insensitive to activation by heparin. To examine this possibility, the zymogen was removed from the rBACE1 preparation by immunoprecipitation with the anti-proBACE1 antibody. The supernatant fraction after immunoprecipitation was enriched in a 65-68 kDa band that lacked immunoreactivity to the anti-proBACE1 antibody (Fig. 6). Removal of the pro-enzyme was found to abolish the stimulatory effect of heparin (1 μg/mL). In contrast, the activity in incubations of the original rBACE1 preparation, which primarily consisted of the zymogen, was strongly stimulated by heparin. As noted previously (Fig. 3), the amount of 70-73 kDa enzyme, which was present in the original preparation and recognised by both the anti-proBACE1 and the EE-17 antibodies, was strongly decreased after incubation with heparin (1 μg/mL) (Fig. 6 B). In contrast, there was no loss of the 65-68 kDa form, which lacked the pro-domain, after incubation with heparin (1 μg/mL). These results indicated that mature rBACE1 lacked the capacity to be activated by heparin and that stimulation of enzyme activity depended on the presence of the pro-domain.

Analysis of rBACE1 autocatalytic cleavage products by mass spectrometry

To identify products of heparin-stimulated autocatalytic cleavage of BACE1, MALDI-TOF MS analysis was performed. rBACE1 was activated by heparin for 10 min in sodium acetate buffer (pH 4) at 37 °C. A sample from this incubation and samples from control incubations containing rBACE1 or heparin alone were then subjected to MALDI-TOF MS analysis.

A number of autocatalytic cleavage products were identified by this method (Fig. 7). However, a peak corresponding to the mass (2536 Da) of the intact pro-peptide which would have been produced by cleavage of the zymogen on the C-terminal side of arginine-

45 was unable to be identified(Fig. 7 A).

Two major peaks at m/z ratio of 1607 and 1841 in the rBACE1 sample incubated with heparin, which were not found in the control incubations were observed (Fig. 7 A). To identify the peptides corresponding to the 1607 and 1841 peaks, the peptides in each fraction were subjected to MSMS analysis. The product ion spectrum of peak 1607 strongly suggested that this peptide was BACE1(185-198), which would have been generated by cleavage at a known autocatalytic cleavage site on the C-terminal side of tyrosine-184 (Lin *et al.*, 2000, *supra*) and at a novel cleavage site on the C-terminal side of phenylalanine-198 (Fig. 7 A, inset; C and D). Although fewer fragments were generated, the product ion spectrum of peak 1841 was consistent with a peptide that was produced by cleavage on the C-terminal side of leucine-182 and phenylalanine-198 i.e., BACE1(183-198) (Fig. 7 A, inset; C and D).

A peak with the m/z ratio of 2084 was also present in samples containing rBACE1 alone i.e., it was not specifically generated by heparin treatment. One peak in the lower molecular mass range that was found in the incubation of rBACE1 with heparin (m/z ratio of 1953) remained to be identified.

In the higher molecular mass range, two peaks at m/z ratio of 5922 and 8441 that were found only in incubations of rBACE1 with heparin were observed (Fig. 7 B). The observed masses were consistent with the masses of the N-terminal product of mature- and proBACE1, respectively, when cleaved at another known autocatalytic cleavage site on the C-terminal side of phenylalanine-99 (Lin *et al.*, 2000, *supra*) (Fig. 7 B-D).

For the 70-73 kDa zymogen, the cleavage at phenylalanine-99 would result in removal of an N-terminal fragment of 8.4 kDa, producing a C-terminal fragment of rBACE1 with an apparent molecular mass of approximately 62-65 kDa. In support of this cleavage, a 62 kDa C-terminal fragment recognised by the anti-His antibody was observed after incubation with heparin (1 μg/mL) (Fig. 3 E). This 62 kDa fragment was not recognised by the N-terminal antibody directed against amino acids 46-62 of BACE1.

Effect of Heparin disaccharide I-S on BACE1 activity

BACE1 activity was measured using a quenched-fluorescence assay. Wells of a black 96-well plate each contained 0.1M sodium acetate buffer at pH 4, 5 % dimethyl-sulfoxide (DMSO), 1 mM substrate peptide, 1 µl of rBACE1. In addition, 10µl of heparin disaccharide analogue (100 µg/ml) and/or 10 µl of heparin (10 µg/ml) was added to some wells. The final volume in each well was 100 µl. Enzyme activity in each well was calculated from the increase in fluorescence intensity (F), at 37°C and was detected using a fluorescence microplate reader (FLUOstar). Excitation and emission wavelengths were set at 320 and 405 nm, respectively. Heparin activity (or inhibition) by test compound addition was determined through analysis of the change in fluorescence over time (ΔF/min). Values shown are means of 3 determinations for each incubation condition.

The results are depicted in Figure 9.

Still another further aspect of the present invention is directed to a method for the treatment and/or prophylaxis of a condition characterised by impaired tissue viability and/or functioning, which impaired tissue viability and/or functioning is directly or indirectly induced by amyloidosis, said method comprising administering to said subject a fragment of heparin or a pharmaceutically acceptable salt thereof.

Yet still another further aspect of the present invention provides a method for the treatment and/or prophylaxis of a condition characterised by encephalon Aβ deposition, said method comprising administering to said subject a fragment of heparin of a pharmaceutically acceptable salt thereof.

Another further aspect of the invention is directed to a method of inhibiting or downregulating BACE1 zymogen mediated functional activity, said method comprising contacting said BACE1 zymogen with a fragment of heparin of a pharmaceutically acceptable salt thereof.

A further aspect of the present invention is directed to a method of modulating the viability and/or functioning of amyloidogenic tissue in a subject, said method comprising administering to said subject a fragment of heparin of a pharmaceutically acceptable salt thereof.

In particularly preferred embodiments the fragment of heparin is a disaccharide compound of Formula (I).

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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CLAIMS:

1. A method of inhibiting or downregulating BACE1 zymogen mediated functional activity, said method comprising contacting said BACE1 zymogen with a fragment of heparin or derivative thereof or a pharmaceutically acceptable salt thereof for a time and under conditions sufficient for said fragment of heparin or derivative thereof or pharmaceutically acceptable salt to antagonise the functional interactivity of heparin or heparin sulphate with said BACE1 zymogen pro-domain.

2. The method according to claim 1 wherein said functional activity is β amyloid precursor protein cleavage.

3. The method according to claim 2 wherein said β amyloid precursor protein cleavage is Aβ production.

4. The method according to any one of claims 1 to 3 wherein said fragment of heparin or derivative thereof or pharmaceutically acceptable salt antogises the functional interactivity of heparin or heparin sulphate with one or more basic amino acids of the BACE1 zymogen pro-domain.

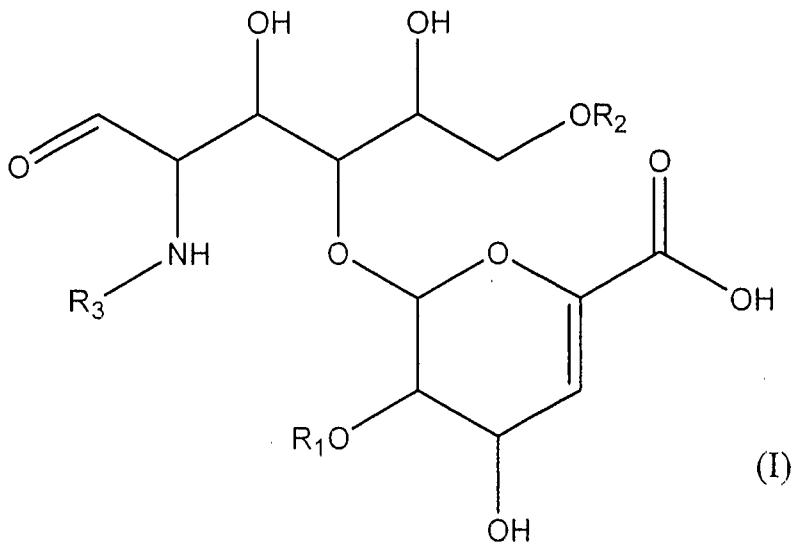
5. The method according to claim 4 wherein said basic amino acids are H24, R27, R31, R42 and R45.

6. The method according to any one of claims 1 to 5, wherein said heparin derivative is a chemically modified form.

7. The method according to claim 6 wherein said chemically modified form is selected from oversulfated heparin, carboxyl-reduced heparin, periodate-oxidized heparin, fully de-O-sulfated heparin, 2-O-desulfated heparin, 6-O-desulfated heparin, fully N-acetylated heparin, fully N-sulfated heparin, de-N-sulfated heparin or de-N-acetylated heparin.

8. The method according to any one of claims 1 to 5 wherein said heparin is a heparin fragment.

9. The method according to claim 8 wherein said heparin fragment is a disaccharide of Formula (I):



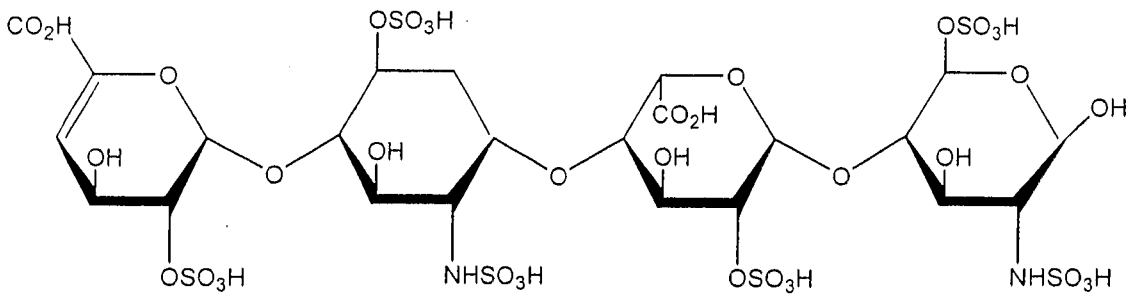
(I)

Heparin disaccharide I-A (ΔUA-2S-[1→ 4]-GlcNAc-6S)	$R_1 = SO_3H$,	$R_2 = SO_3H$,	$R_3 = Ac$
Heparin disaccharide I-H (ΔUA-2S-[1→ 4]-GlcN-6S)	$R_1 = SO_3H$,	$R_2 = SO_3H$,	$R_3 = H$
Heparin disaccharide I-S (ΔUA-2S-[1→ 4]-GlcNS-6S)	$R_1 = SO_3H$,	$R_2 = SO_3H$,	$R_3 = SO_3H$
Heparin disaccharide II-A (ΔUA-[1→ 4]-GlcNAc-6S)	$R_1 = H$,	$R_2 = SO_3H$,	$R_3 = Ac$
Heparin disaccharide II-H (ΔUA-[1→ 4]-GlcN-6S)	$R_1 = H$,	$R_2 = SO_3H$,	$R_3 = H$
Heparin disaccharide II-S (ΔUA-[1→ 4]-GlcNS-6S)	$R_1 = H$,	$R_2 = SO_3H$,	$R_3 = SO_3H$
Heparin disaccharide III-A	$R_1 = SO_3H$,	$R_2 = H$,	$R_3 = Ac$

(ΔUA-2S[1→ 4]-GlcNAc)			
Heparin disaccharide III-H	$R_1 = SO_3H$,	$R_2 = H$,	$R_3 = H$
(ΔUA-2S[1→ 4]-GlcN)			
Heparin disaccharide III-S	$R_1 = SO_3H$,	$R_2 = H$,	$R_3 = SO_3H$
(ΔUA-2S[1→ 4]-GlcNS)			
Heparin disaccharide IV-S	$R_1 = H$,	$R_2 = H$,	$R_3 = SO_3H$
(ΔUA-[1→ 4]-GlcNS)			

10. The method according to claim 9 wherein said disaccharide compound is α-ΔUA-25-[1→ 4]-GlcNS-6S (Heparin disaccharide I-S).

11. The method according to claim 8 wherein said heparin fragment is selected from:
Heparin tetrasaccharide, HexA,2S-GlcNS,6S-(IdoA,2S-GlcNS,6S)



Heparin hexasaccharide, HexA,2S-GlcNS,6S-(IdoA,2S-GlcNS,6S)$_2$
Heparin octasaccharide, HexA,2S-GlcNS,6S-(IdoA,2S-GlcNS,6S)$_3$
Heparin decasaccharide, HexA,2S-GlcNS,6S-(IdoA,2S-GlcNS,6S)$_4$
Heparin dodecasaccharide, HexA,2S-GlcNS,6S-(IdoA,2S-GlcNS,6S)$_5$
Heparin tetradecasaccharide, HexA,2S-GlcNS,6S-(IdoA,2S-GlcNS,6S)$_6$.

12. A method of modulating the viability and/or functioning of Aβ amyloidogenic tissue in a subject, said method comprising administering to said subject an effective amount of a fragment of heparin or derivative thereof or a pharmaceutically acceptable salt thereof for a time and under conditions sufficient for said fragment of heparin or derivative

thereof or pharmaceutically acceptable salt to antagonise the functional interactivity of heparan sulphate with the BACE1 zymogen pro-domain wherein antagonising said interaction inhibits or downregulates BACE1 zymogen mediated β amyloid precursor protein cleavage.

13. A method for the treatment and/or prophylaxis of a condition characterised by impaired tissue viability and/or functioning, which impaired tissue viability and/or functioning is directly or indirectly induced by Aβ amyloidosis, said method comprising administering to said subject an effective amount of a fragment of heparin or derivative thereof or a pharmaceutically acceptable salt thereof for a time and under conditions sufficient for said fragment of heparin or derivative thereof or pharmaceutically acceptable salt to antagonise the functional interactivity of heparin sulphate with the BACE1 zymogen pro-domain wherein antagonising said interaction inhibits or downregulates BACE1 zymogen mediated β amyloid precursor protein cleavage.

14. The method according to claim 12 or 13 wherein said tissue is encephalon tissue.

15. The method according to claim 14 wherein modulation of the viability and/or functioning of Aβ amyloidogenic encephalon tissue is upregulation of Aβ amyloidogenic encephalon cognitive functioning.

16. The method according to claim 15 wherein condition is characterised by Aβ deposition.

17. The method according to claim 16 wherein said condition is Alzheimer's Disease, Down's Syndrome, cerebral haemorrhage with Aβ amyloidosis, Aβ amyloidosis associated with chronic inflammation or β amyloidosis associated with senile cardiac amyloid.

18. Use of a fragment of heparin or derivative thereof or a pharmaceutically acceptable salt thereof in the manufacture of a medicament for the prophylactic and/or therapeutic treatment of a condition characterised by impaired tissue viability and/or

functioning, which impaired viability and/or functioning is directly or indirectly induced by Aβ amyloidosis, wherein said fragment of heparin or derivative thereof or pharmaceutically acceptable salt antagonises the functional interactivity of heparin sulphate with the BACE1 zymogen pro-domain.

19. Use according to claim 18 wherein said condition is characterised by Aβ deposition.

20. Use according to claim 19 wherein said tissue is encephalon tissue.

21. Use according to claim 20 wherein said condition is Alzheimer's Disease, Down's Syndrome, cerebral haemorrhage with Aβ amyloidosis, Aβ amyloidosis associated with chronic inflammation or β amyloidosis associated with senile cardiac amyloid.

22. The method according to claims 12 to 17 or the use according to claims 18 to 21 wherein said functional activity is β amyloid precursor protein cleavage.

23. The method or use according to claim 22 wherein said β amyloid precursor protein cleavage is Aβ production.

24. The method or use according to any one of claims 12 to 23 wherein said fragment of heparin or derivative thereof or pharmaceutically acceptable salt antogises the functional interactivity of heparin or heparin sulphate with one or more basic amino acids of the BACE1 zymogen pro-domain.

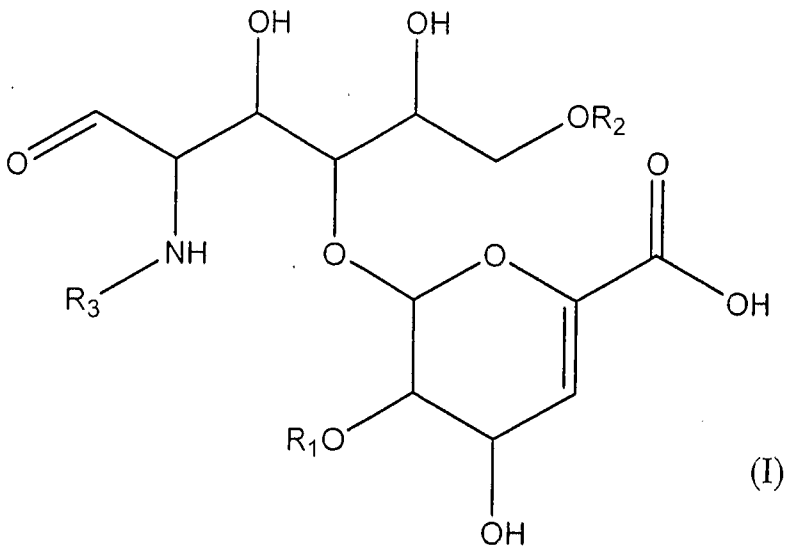
25. The method or use according to claim 24 wherein said basic amino acids are H24, R27, R31, R42 and R45.

26. The method or use according to claims 12 to 25 wherein said heparin derivative is a chemically modified form.

27. The method of use according to claim 26 wherein said chemically modified form is selected from oversulfated heparin, carboxyl-reduced heparin, periodate-oxidized heparin, fully de-O-sulfated heparin, 2-O-desulfated heparin, 6-O-desulfated heparin, fully N-acetylated heparin, fully N-sulfated heparin, de-N-sulfated heparin or de-N-acetylated heparin.

28. The method or use according to claims 12 to 25 wherein said heparin is a heparin fragment.

29. The method or use according to claim 28 wherein said heparin fragment is a disaccharide of Formula (I):



(I)

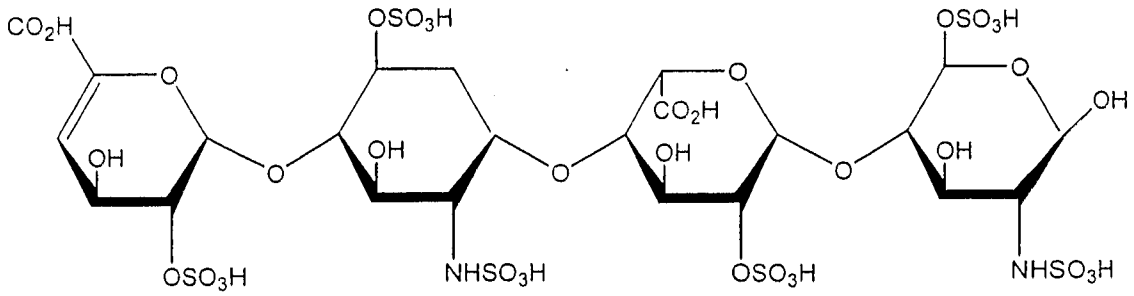
Heparin disaccharide I-A (ΔUA-2S-[1→ 4]-GlcNAc-6S)	$R_1 = SO_3H$,	$R_2 = SO_3H$,	$R_3 = Ac$
Heparin disaccharide I-H (ΔUA-2S-[1→ 4]-GlcN-6S)	$R_1 = SO_3H$,	$R_2 = SO_3H$,	$R_3 = H$
Heparin disaccharide I-S (ΔUA-2S-[1→ 4]-GlcNS-6S)	$R_1 = SO_3H$,	$R_2 = SO_3H$,	$R_3 = SO_3H$
Heparin disaccharide II-A (ΔUA-[1→ 4]-GlcNAc-6S)	$R_1 = H$,	$R_2 = SO_3H$,	$R_3 = Ac$

Heparin disaccharide II-H (ΔUA-[1→ 4]-GlcN-6S)	R_1 = H,	R_2 = SO_3H,	R_3 = H
Heparin disaccharide II-S (ΔUA-[1→ 4]-GlcNS-6S)	R_1 = H,	R_2 = SO_3H,	R_3 = SO_3H
Heparin disaccharide III-A (ΔUA-2S[1→ 4]-GlcNAc)	R_1 = SO_3H,	R_2 = H,	R_3 = Ac
Heparin disaccharide III-H (ΔUA-2S[1→ 4]-GlcN)	R_1 = SO_3H,	R_2 = H,	R_3 = H
Heparin disaccharide III-S (ΔUA-2S[1→ 4]-GlcNS)	R_1 = SO_3H,	R_2 = H,	R_3 = SO_3H
Heparin disaccharide IV-S (ΔUA-[1→ 4]-GlcNS).	R_1 = H,	R_2 = H,	R_3 = SO_3H

30. The method or use according to claim 29 wherein said disaccharide compound is α-ΔUA-25-[1→ 4]-GlcNS-6S (Heparin disaccharide I-S).

31. The method or use according to claim 27 wherein said heparin fragment is selected from:

Heparin tetrasaccharide, HexA,2S-GlcNS,6S-(IdoA,2S-GlcNS,6S)



Heparin hexasaccharide, HexA,2S-GlcNS,6S-(IdoA,2S-GlcNS,6S)$_2$

Heparin octasaccharide, HexA,2S-GlcNS,6S-(IdoA,2S-GlcNS,6S)$_3$

Heparin decasaccharide, HexA,2S-GlcNS,6S-(IdoA,2S-GlcNS,6S)$_4$

Heparin dodecasaccharide, HexA,2S-GlcNS,6S-(IdoA,2S-GlcNS,6S)$_5$

Heparin tetradecasaccharide, HexA,2S-GlcNS,6S-(IdoA,2S-GlcNS,6S)$_6$.

Figure 1

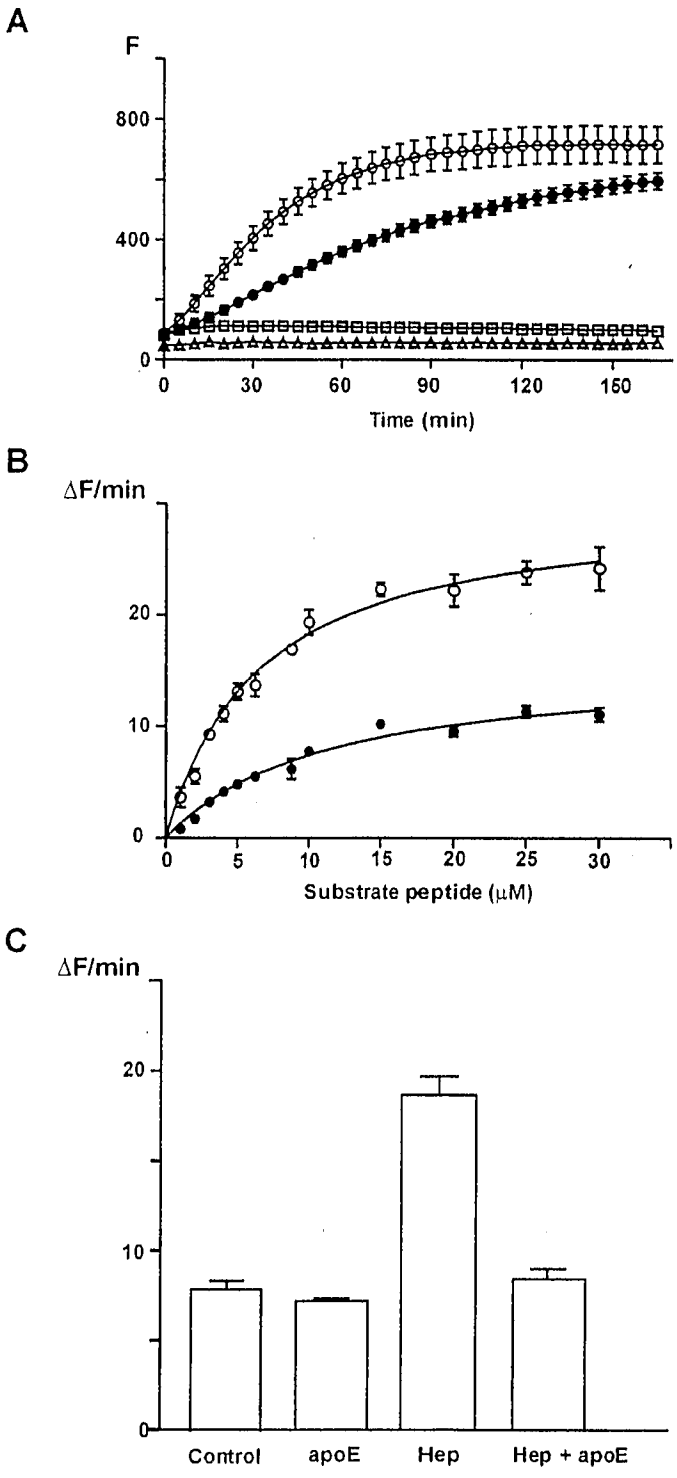

A
F
800
400
0
0
30
60
90
120
150
Time (min)
B
ΔF/min
20
10
0
0
5
10
15
20
25
30
Substrate peptide (μM)
C
ΔF/min
20
10
0
Control
apoE
Hep
Hep + apoE

Figure 2

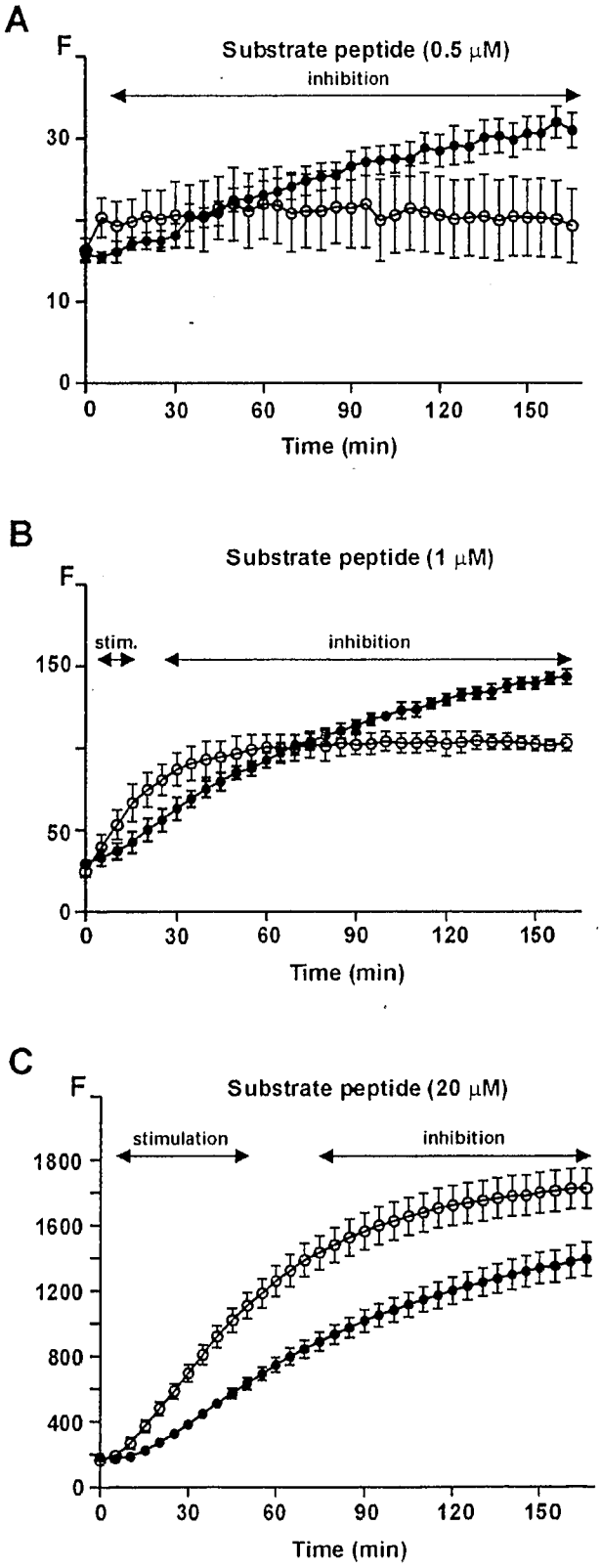

A
F
Substrate peptide (0.5 μM)
inhibition
30
10
0
0
30
60
90
120
150
Time (min)
B
F
Substrate peptide (1 μM)
stim.
inhibition
150
50
0
0
30
60
90
120
150
Time (min)
C
F
Substrate peptide (20 μM)
stimulation
inhibition
1800
1600
1200
800
400
0
0
30
60
90
120
150
Time (min)

Figure 3

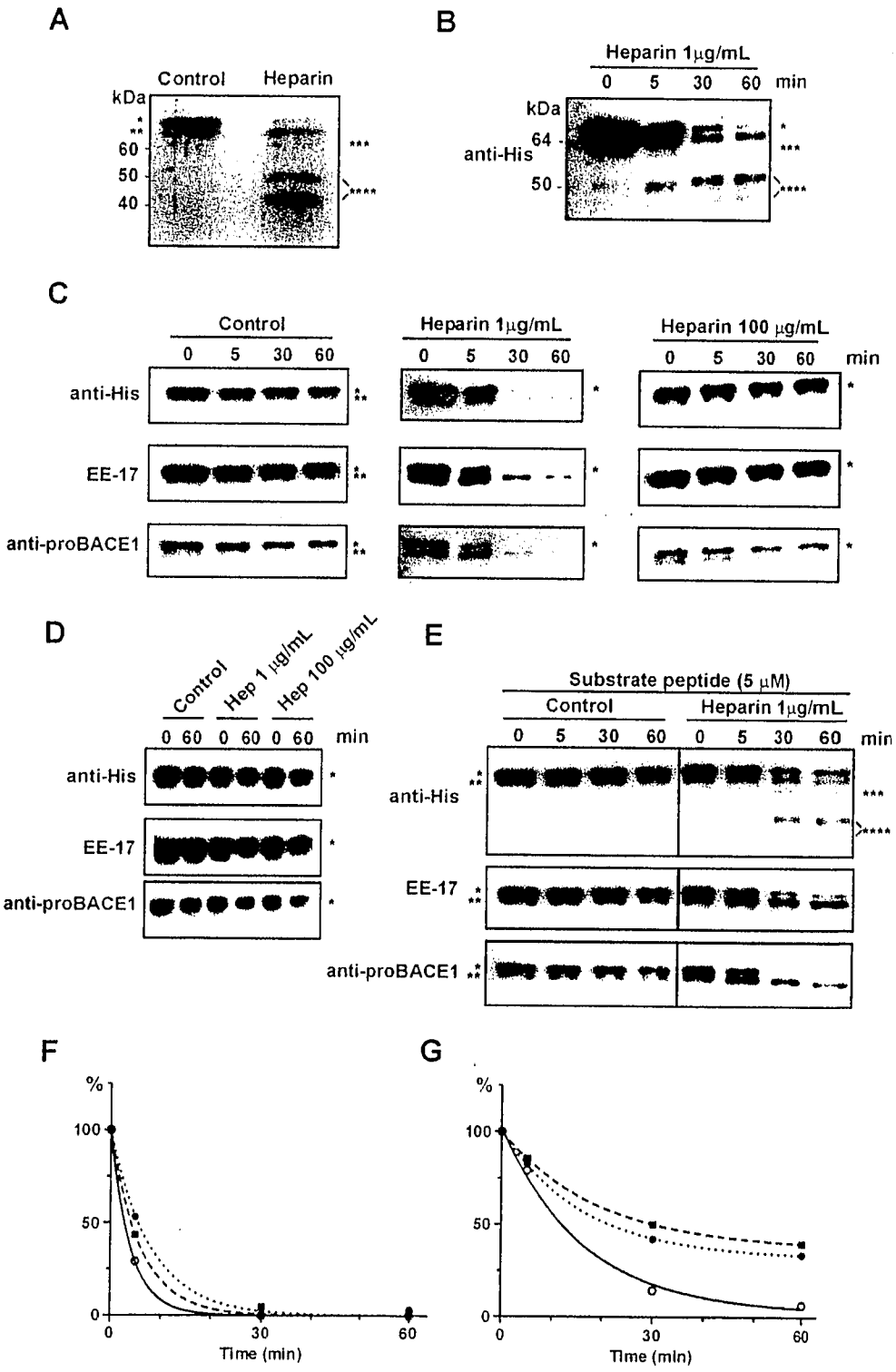

A
Control
Heparin
kDa
60
50
40
B
Heparin 1μg/mL
0 5 30 60 min
kDa
64
anti-His
50
C
Control
Heparin 1μg/mL
Heparin 100 μg/mL
0 5 30 60
min
anti-His
EE-17
anti-proBACE1
D
Control
Hep 1 μg/mL
Hep 100 μg/mL
0 60 0 60 0 60
min
anti-His
EE-17
anti-proBACE1
E
Substrate peptide (5 μM)
Control
Heparin 1μg/mL
0 5 30 60 0 5 30 60
min
anti-His
EE-17
anti-proBACE1
F
%
100
50
0
0
30
60
Time (min)
G
%
100
50
0
0
30
60
Time (min)

Figure 4

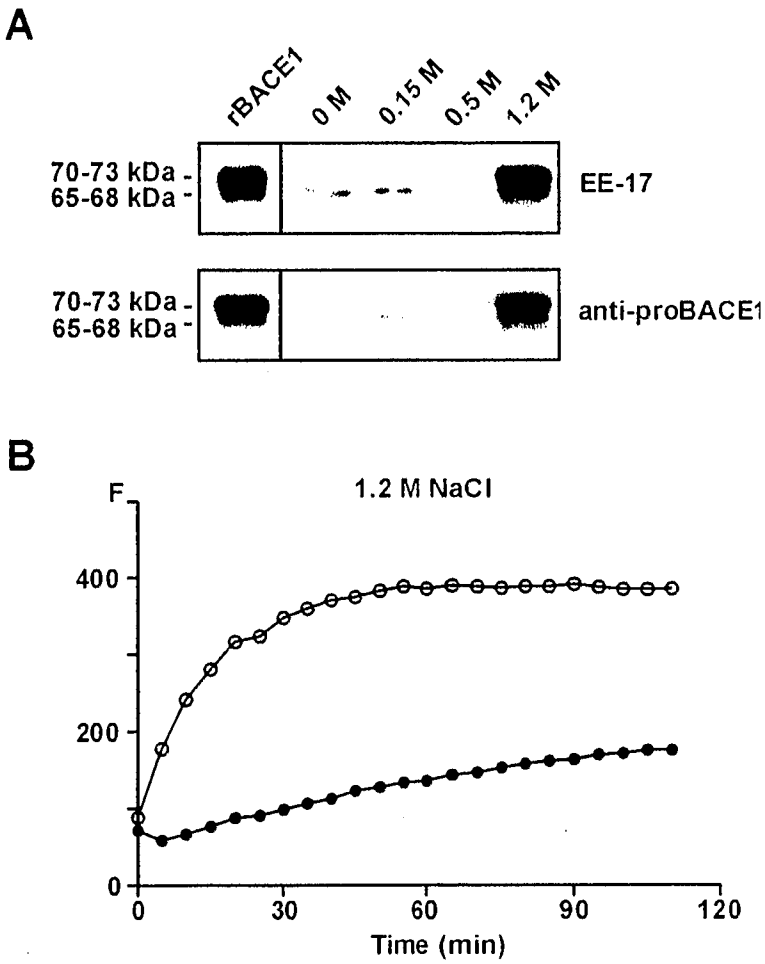

A
rBACE1
0 M
0.15 M
0.5 M
1.2 M
70-73 kDa
65-68 kDa
EE-17
70-73 kDa
65-68 kDa
anti-proBACE1
B
1.2 M NaCl
F
400
200
0
0
30
60
90
120
Time (min)

Figure 5

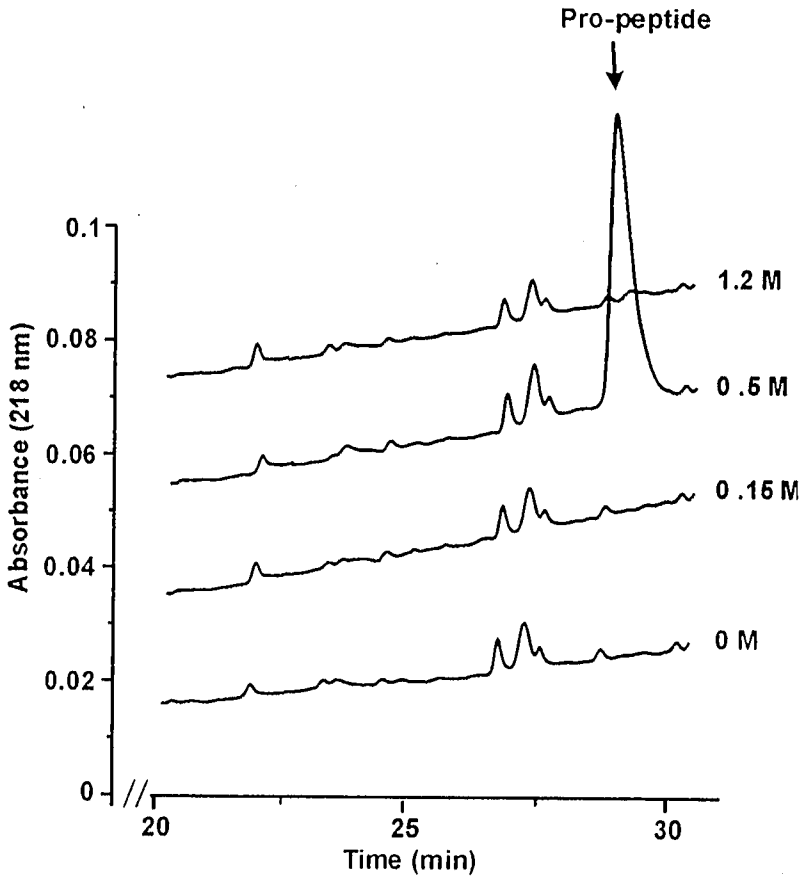
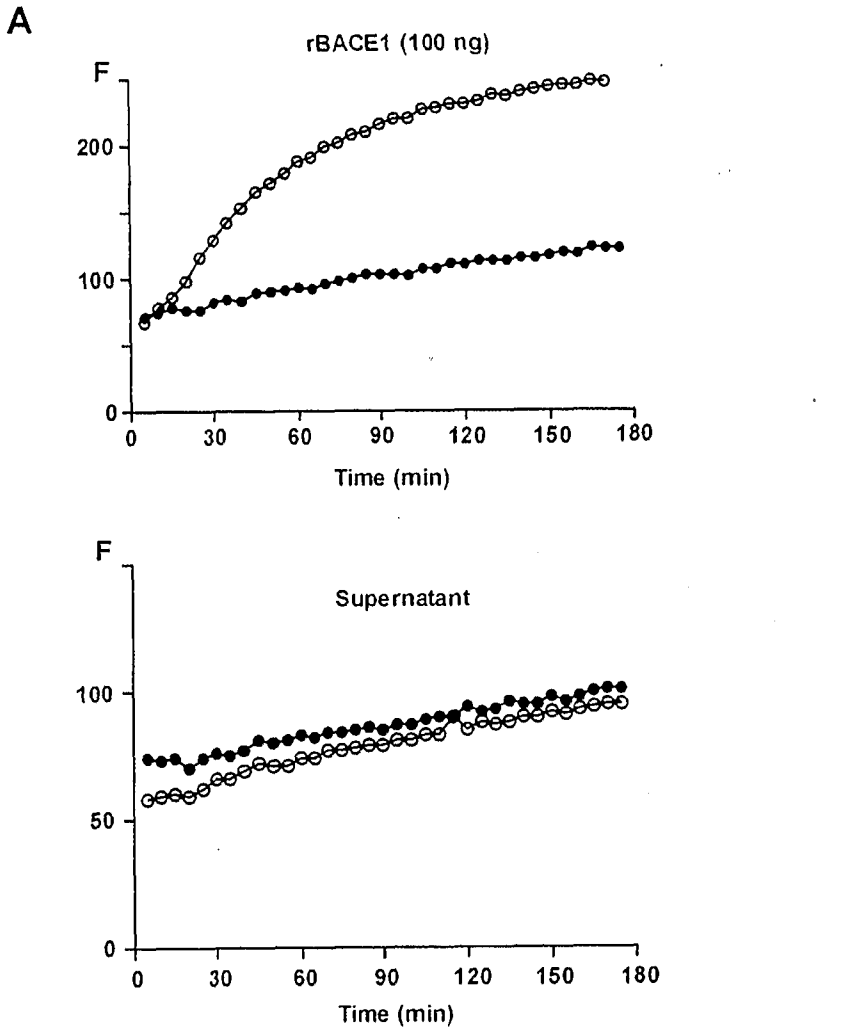

Pro-peptide
1.2 M
0 .5 M
0 .15 M
0 M
Absorbance (218 nm)
0.1
0.08
0.06
0.04
0.02
0
20
25
30
Time (min)

Figure 6


A
rBACE1 (100 ng)
F
200
100
0
0
30
60
90
120
150
180
Time (min)
F
Supernatant
100
50
0
0
30
60
90
120
150
180
Time (min)

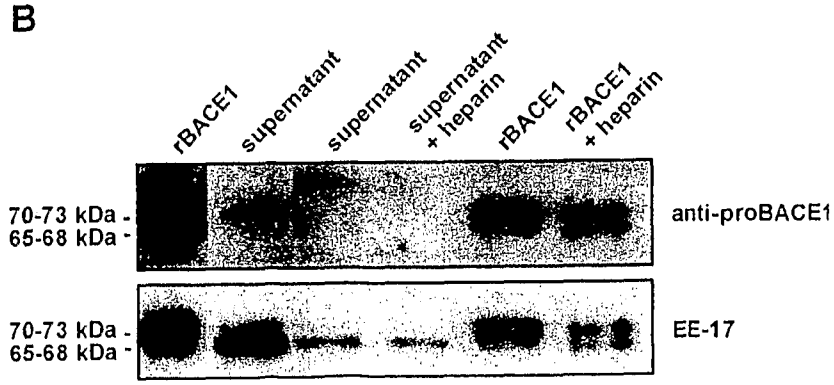

B
rBACE1
supernatant
supernatant
supernatant + heparin
rBACE1
rBACE1 + heparin
70-73 kDa
65-68 kDa
anti-proBACE1
70-73 kDa
65-68 kDa
EE-17

Figure 7A

A

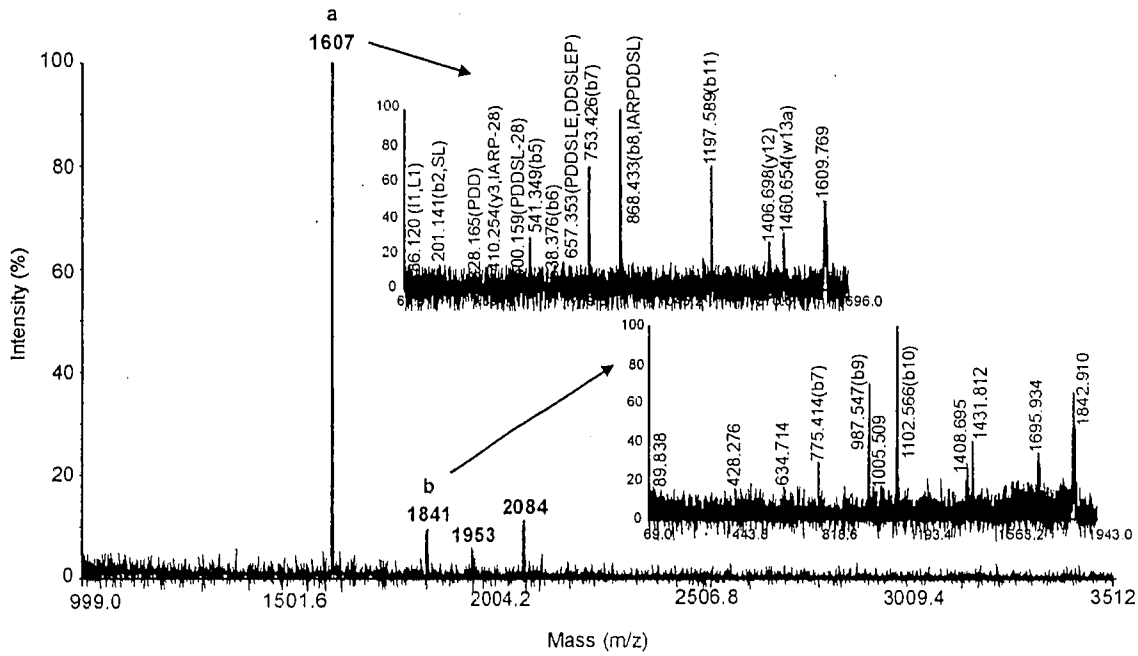
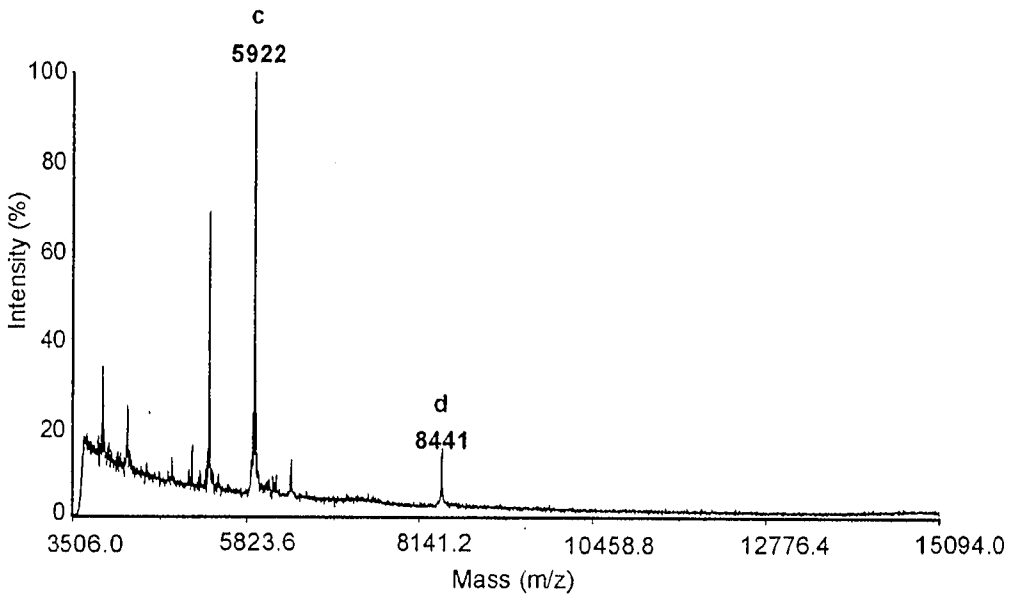

a
1607
b
1841
1953
2084
Intensity (%)
Mass (m/z)
999.0
1501.6
2004.2
2506.8
3009.4
3512
136.120 (l1,L1)
201.141(b2,SL)
328.165(PDD)
410.254(y3,IARP-28)
500.159(PDDSL-28)
541.349(b5)
638.376(b6)
657.353(PDDSLE,DDSLEP)
753.426(b7)
868.433(b8,IARPDDSL)
1197.589(b11)
1406.698(y12)
1460.654(w13a)
1609.769
89.838
428.276
634.714
775.414(b7)
987.547(b9)
1005.509
1102.566(b10)
1408.695
1431.812
1695.934
1842.910

Figure 7B-D

B



C

Peak	Observed mass	Peptide	Theoretical mass
a	1607	BACE1(185-198)	1607
b	1841	BACE1(183-198)	1841
c	5922	BACE1(46-99)	5922
d	8441	BACE1(22-99)	8440

D

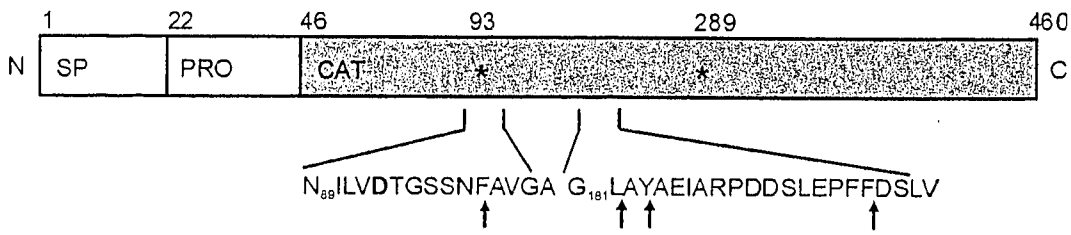


Figure 8

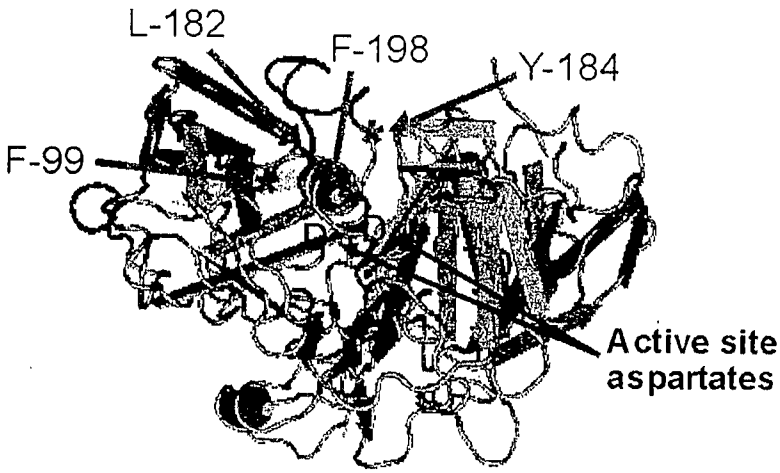

L-182
F-198
Y-184
F-99
Active site
aspartates
* Autocatalytic cleavage sites

Figure 10A

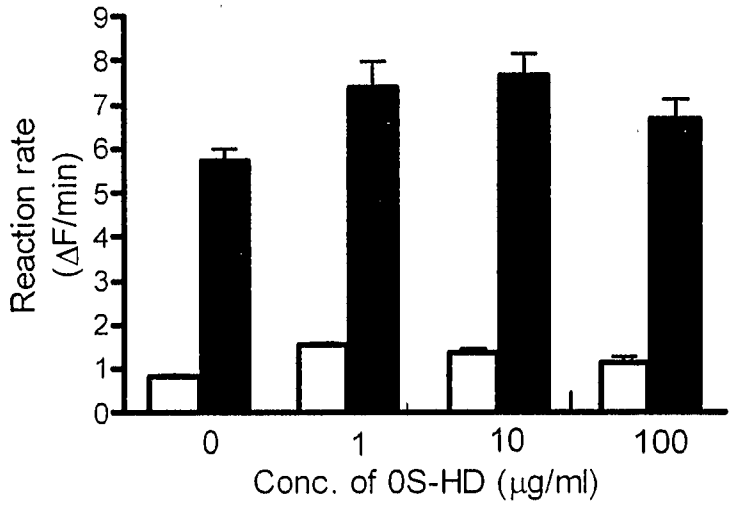


Figure 10B

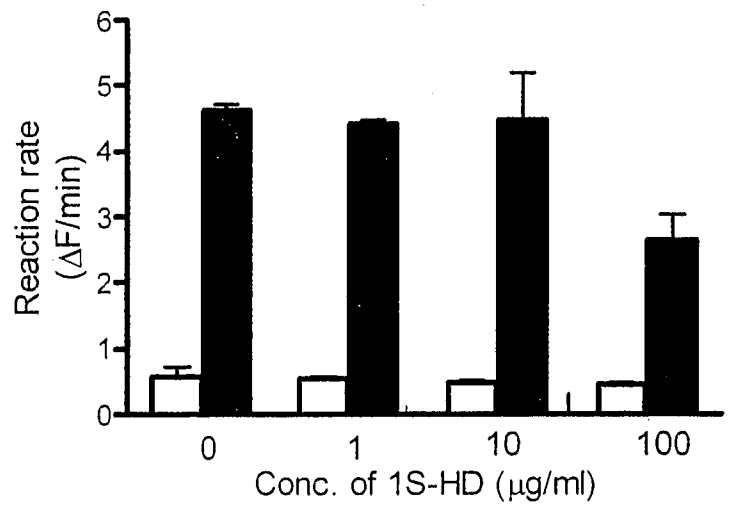


Figure 10C

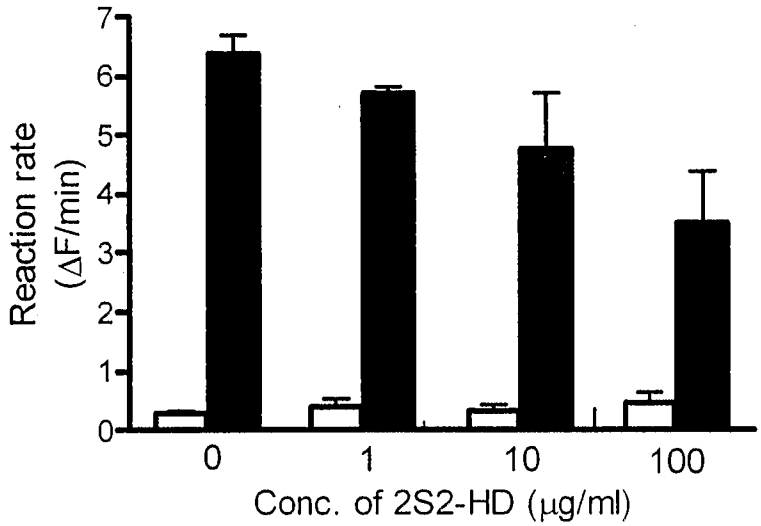


Figure 10D

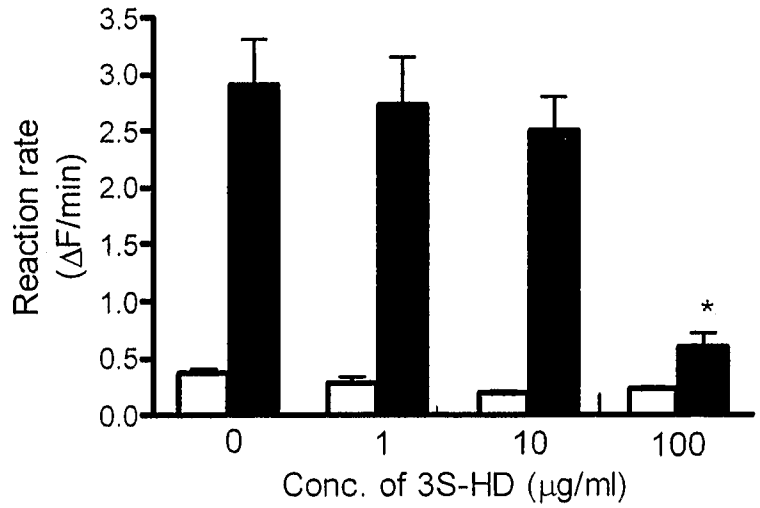


Figure 11A

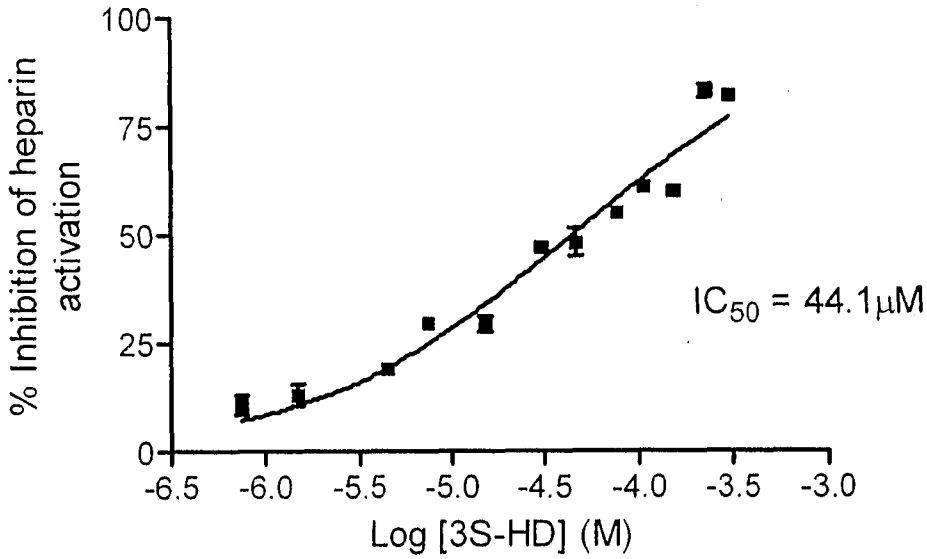


Figure 11B

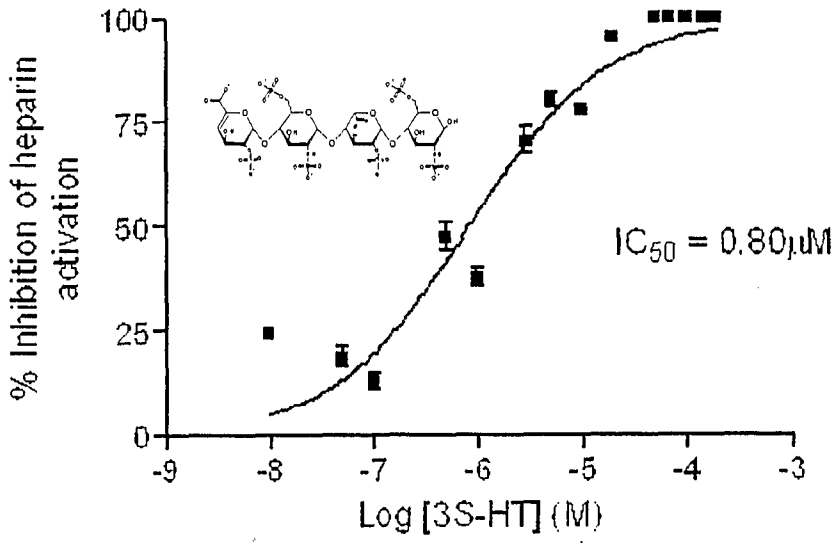


Figure 12A

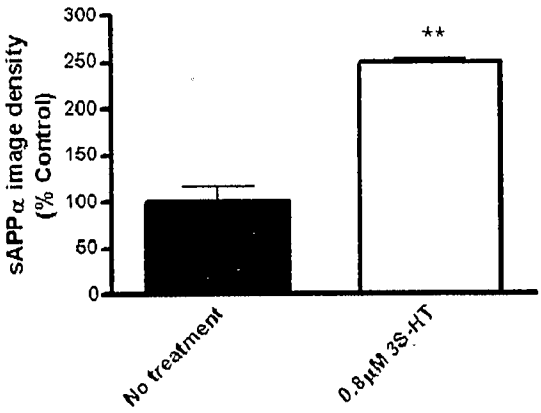


Figure 12B

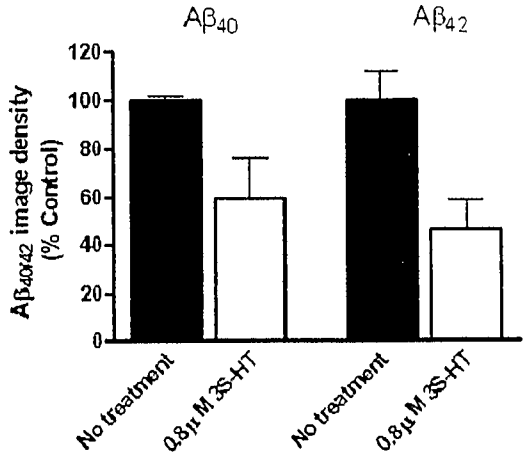
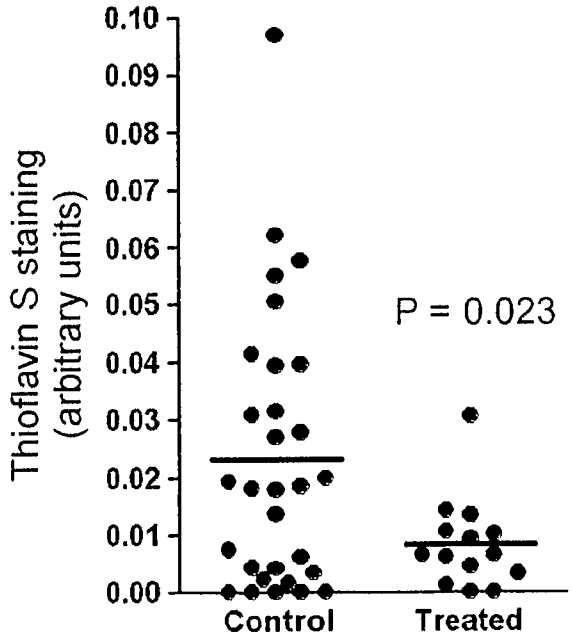


Figure 13



INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2007/000472

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. ***A61P 25/00*** (2006.01), ***A61K 31/727*** (2006.01), ***A61K 31/00*** (2006.01), ***A61P 25/28*** (2006.01)

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)
WPIDS, MEDLINE, CAPLUS; BACE1, Beta-site amyloid precursor protein cleaving enzyme-1, Heparin, Beta-amyloid, Alzheimer's Disease, Down Syndrome

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Scholefield, Z. et al. 2003. Heparan sulfate regulates amyloid precursor protein processing by BACE1, the Alzheimer's β-secretase. The Journal of Cell Biology. 163(1):97-107. (See Fig 1, Fig 4 and page 105 RHS)	1-31
PX	Beckman, M. et al. 2006. Heparin activates β-secretase (BACE1) of Alzheimer's disease and increases autocatalysis of the enzyme. Biochemistry. 45:6703-6714 (See whole document)	1-31
PX	Patey, S.J. et al. 2006. Heparin derivatives as inhibitors of BACE-1, the Alzheimer's β-Secretase, with reduced activity against Factor Xa and other proteases. Journal of Medicinal Chemistry. 49:6129-6132. (See whole document)	1-31
A	Leveugle, B. et al. 1997. Heparin promotes β-secretase cleavage of the Alzheimer's amyloid precursor protein. Neurochemistry International. 30(6):543-548. (See whole document)	1-31

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

* Special categories of cited documents:
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 May 2007	01 MAY 2007
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer **RAMILA DEWALAGAMA** AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No : (02) 6222 3659