

(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
28 December 2006 (28.12.2006)

PCT

(10) International Publication Number
WO 2006/138363 A2(51) International Patent Classification:
G01N 33/53 (2006.01)[US/US]; 126 East Lincoln Avenue, Rahway, New Jersey 07065-0907 (US). **STONE, David, J.** [US/US]; 126 East Lincoln Avenue, Rahway, New Jersey 07065-0907 (US).

(21) International Application Number:

PCT/US2006/023144

(74) Common Representative: **MERCK & CO., INC.**; 126 East Lincoln Avenue, Rahway, New Jersey 07065-0907 (US).

(22) International Filing Date: 14 June 2006 (14.06.2006)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

60/691,693 17 June 2005 (17.06.2005) US
60/694,964 29 June 2005 (29.06.2005) US(71) Applicant (for all designated States except US): **MERCK & CO., INC.** [US/US]; 126 East Lincoln Avenue, Rahway, New Jersey 07065-0907 (US).

(72) Inventors; and

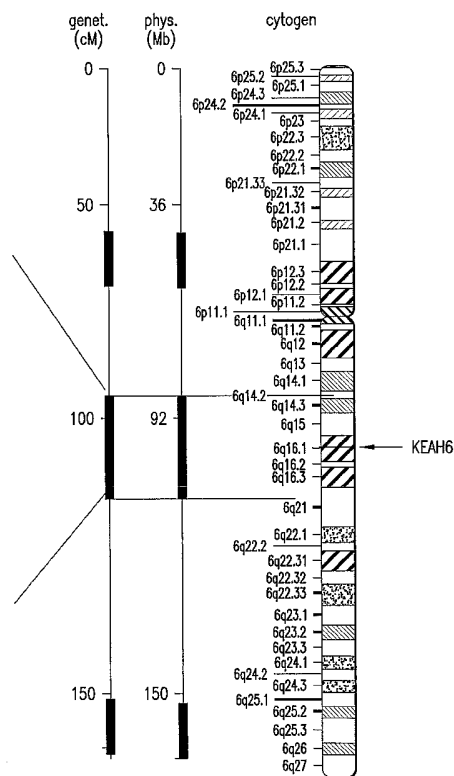
(75) Inventors/Applicants (for US only): **MAJERCAK, John, M.** [US/US]; 126 East Lincoln Avenue, Rahway, New Jersey 07065-0907 (US). **RAY, William, J.**

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI,

[Continued on next page]

(54) Title: METHOD FOR IDENTIFYING MODULATORS OF KEAH6 USEFUL FOR TREATING ALZHEIMER'S DISEASE

(57) Abstract: Methods for identifying modulators of KEAH6 are described. The methods are particularly useful for identifying analytes that antagonize KEAH6's effect on processing of amyloid precursor protein to A β peptide and thus useful for identifying analytes that can be used for treating Alzheimer disease.



FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT,
RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA,
GN, GQ, GW, ML, MR, NE, SN, TD, TG).

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Published:

— *without international search report and to be republished
upon receipt of that report*

TITLE OF THE INVENTION

METHOD FOR IDENTIFYING MODULATORS OF KEAH6

USEFUL FOR TREATING ALZHEIMER'S DISEASE

5 BACKGROUND OF THE INVENTION

(1) Field of the Invention

10 The present invention relates to methods for identifying modulators of KEAH6. The methods are particularly useful for identifying analytes that antagonize KEAH6's effect on processing of amyloid precursor protein to A β peptide and thus useful for identifying analytes that can be used for treating Alzheimer disease.

(2) Description of Related Art

15 Alzheimer's disease is a common, chronic neurodegenerative disease, characterized by a progressive loss of memory and sometimes severe behavioral abnormalities, as well as an impairment of other cognitive functions that often leads to dementia and death. It ranks as the fourth leading cause of death in industrialized societies after heart disease, cancer, and stroke. The incidence of Alzheimer's disease is high, with an estimated 2.5 to 4 million patients affected in the United States and perhaps 17 to 25 million worldwide. Moreover, the number of sufferers is expected to grow as the population ages.

20 A characteristic feature of Alzheimer's disease is the presence of large numbers of insoluble deposits, known as amyloid plaques, in the brains of those affected. Autopsies have shown that amyloid plaques are found in the brains of virtually all Alzheimer's patients and that the degree of amyloid plaque deposition often correlates with the degree of dementia (Cummings and Cotman, Lancet 326:1524-1587 (1995)). While some opinion holds that amyloid plaques are a late stage by-product of the disease process, the consensus view is that amyloid plaques and/or soluble aggregates of amyloid peptides are more likely to be intimately, and perhaps causally, involved in Alzheimer's disease.

25 A variety of experimental evidence supports this view. For example, amyloid β (A β) peptide, a primary component of amyloid plaques, is toxic to neurons in culture and transgenic mice that overproduce A β peptide in their brains show extensive deposition of A β into amyloid plaques as well as significant neuronal toxicity (Yankner, Science 250: 279-282 (1990); Mattson *et al.*, J. Neurosci. 12: 379-389 (1992); Games *et al.*, Nature 373: 523-527 (1995); LaFerla *et al.*, Nature Genetics 9: 21-29 (1995)). Mutations in the APP gene, leading to increased A β production, have been linked to heritable forms of Alzheimer's disease (Goate *et al.*, Nature 349:704-706 (1991); Chartier-Harlan *et al.*, Nature 353:844-846 (1991); Murrel *et al.*, Science 254: 97-99 (1991); Mullan *et al.*, Nature Genetics 1: 345-347 (1992)). Presenilin-1 (PS1) and presenilin-2 (PS2) related familial early-onset Alzheimer's disease (FAD) shows disproportionately increased production of A β 1-42, the 42 amino acid isoform of A β , as opposed to A β 1-40, the 40 amino acid isoform (Scheuner *et al.*, Nature Medicine 2: 864-870 (1996)).

The longer isoform of A β is more prone to aggregation than the shorter isoform (Jarrett *et al.*, Biochemistry 32: 4693-4697 (1993)). Injection of the insoluble, fibrillar form of A β into monkey brains results in the development of pathology (neuronal destruction, tau phosphorylation, microglial proliferation) that closely mimics Alzheimer's disease in humans (Geula *et al.*, Nature Medicine 4:827-831 (1998)). See Selkoe, J. Neuropathol. Exp. Neurol. 53: 438-447 (1994) for a review of the evidence that amyloid plaques have a central role in Alzheimer's disease.

A β peptide, a 39-43 amino acid peptide derived by proteolytic cleavage of the amyloid precursor protein (APP), is the major component of amyloid plaques (Glennner and Wong, Biochem. Biophys. Res. Comm. 120:885- 890 (1984)). APP is actually a family of polypeptides produced by alternative splicing from a single gene. Major forms of APP are known as APP695, APP751, and APP770, with the subscripts referring to the number of amino acids in each splice variant (Ponte *et al.*, Nature 331: 525-527 (1988); Tanzi *et al.*, Nature 331: 528-530 (1988); Kitaguchi *et al.*, Nature 331: 530-532(1988)). APP is a ubiquitous membrane-spanning (type 1) glycoprotein that undergoes proteolytic cleavage by at least two pathways (Selkoe, Trends Cell Biol. 8: 447-453 (1998)). In one pathway, cleavage by an enzyme known as α -secretase occurs while APP is still in the trans-Golgi secretory compartment (Kuentzel *et al.*, Biochem. J. 295:367-378 (1993)). This cleavage by α - secretase occurs within the A β peptide portion of APP, thus precluding the formation of A β peptide. In an alternate proteolytic pathway, cleavage of the Met596-Asp597 bond (numbered according to the 695 amino acid protein) by an enzyme known as β -secretase occurs. This cleavage by β -secretase generates the N-terminus of A β peptide. The C-terminus is formed by cleavage by a second enzyme known as γ -secretase. The C-terminus is actually a heterogeneous collection of cleavage sites rather than a single site since γ -secretase activity occurs over a short stretch of APP amino acids rather than at a single peptide bond. Peptides of 40 or 42 amino acids in length (A β 1-40 and A β 1-42, respectively) predominate among the C-termini generated by γ -secretase. A β 1-42 peptide is more prone to aggregation than A β 1-40 peptide, the major secreted species (Jarrett *et al.*, Biochemistry 32: 4693-4697 91993); Kuo *et al.*, J. Biol. Chem. 271:4077-4081 (1996)), and its production is closely associated with the development of Alzheimer's disease (Sinha and Lieberburg, Proc. Natl. Acad. Sci. USA 96: 11049-11053 (1999)). The bond cleaved by γ -secretase appears to be situated within the transmembrane domain of APP. For a review that discusses APP and its processing, see Selkoe, Trends Cell. Biol. 8:447-453 (1998).

While abundant evidence suggests that extracellular accumulation and deposition of A β peptide is a central event in the etiology of Alzheimer's disease, recent studies have also proposed that increased intracellular accumulation of A β peptide or amyloid containing C-terminal fragments may play a role in the pathophysiology of Alzheimer's disease. For example, over-expression of APP harboring mutations which cause familial Alzheimer's disease results in the increased intracellular accumulation of C100 in neuronal cultures and A β 42 peptide in HEK 293 cells. Moreover, evidence suggests that intra-

and extracellular A β peptide are formed in distinct cellular pools in hippocampal neurons and that a common feature associated with two types of familial Alzheimer's disease mutations in APP ("Swedish" and "London") is an increased intracellular accumulation of AP42 peptide. Thus, based on these studies and earlier reports implicating extracellular A β peptide accumulation in Alzheimer's disease pathology, it appears that altered APP catabolism may be involved in disease progression.

Much interest has focused on the possibility of inhibiting the development of amyloid plaques as a means of preventing or ameliorating the symptoms of Alzheimer's disease. To that end, a promising strategy is to inhibit the activity of β - and γ - secretase, the two enzymes that together are responsible for producing A β . This strategy is attractive because, if the formation of amyloid plaques is a result of the deposition of A β is a cause of Alzheimer's disease, inhibiting the activity of one or both of the two secretases would intervene in the disease process at an early stage, before late- stage events such as inflammation or apoptosis occur. Such early stage intervention is expected to be particularly beneficial (see, for example, Citron, Molecular Medicine Today 6:392-397 (2000)).

To that end, various assays have been developed that are directed to the identification of substances that may interfere with the production of A β peptide or its deposition into amyloid plaques. U.S. Patent No. 5,441,870 is directed to methods of monitoring the processing of APP by detecting the production of amino terminal fragments of APP. U.S. Patent No. 5,605,811 is directed to methods of identifying inhibitors of the production of amino terminal fragments of APP. U.S. Patent No. 5,593,846 is directed to methods of detecting soluble A β by the use of binding substances such as antibodies. US Published Patent Application No. US20030200555 describes using amyloid precursor proteins with modified β -secretase cleavage sites to monitor beta-secretase activity. Esler *et al.*, Nature Biotechnology 15: 258-263 (1997) described an assay that monitored the deposition of A β peptide from solution onto a synthetic analogue of an amyloid plaque. The assay was suitable for identifying substances that could inhibit the deposition of A β peptide. However, this assay is not suitable for identifying substances, such as inhibitors of β - or γ -secretase, that would prevent the formation of A β peptide.

Various groups have cloned and sequenced cDNA encoding a protein that is believed to be β -secretase (Vassar *et al.*, Science 286: 735-741 (1999); Hussain *et al.*, Mol. Cell. Neurosci. 14: 419-427 (1999); Yan *et al.*, Nature 402: 533-537 (1999); Sinha *et al.*, Nature 402: 537-540 (1999); Lin *et al.*, Proc. Natl. Acad. Sci. USA 97: 1456-1460 (2000)). U.S. Pat. Nos. 6,828,117 and 6,737,510 disclose a β -secretase, which the inventors call aspartyl protease 2 (Asp2), variant Asp-2(a) and variant Asp-2(b), respectively, and U.S. Pat. No. 6,545,127 discloses a catalytically active enzyme known as memapsin. Hong *et al.*, Science 290: 150-153 (2000) determined the crystal structure of the protease domain of human β -secretase complexed with an eight- residue peptide- like inhibitor at 1.9 angstrom resolution. Compared to other human aspartic proteases, the active site of human β -secretase is more open and less hydrophobic, contributing to the broad substrate specificity of human β -secretase (Lin *et al.*, Proc. Natl. Acad. Sci. USA 97:1456-1460 (2000)).

Ghosh *et al.*, *J. Am. Chem. Soc.* 122:3522-3523 (2000) disclosed two inhibitors of β -secretase, OM99-1 and OM99-2, that are modified peptides based on the β -secretase cleavage site of the Swedish mutation of APP (SEVNL/DAEFR, with "/" indicating the site of cleavage). OM99-1 has the structure VNL*AAEF (with "L*A" indicating the uncleavable hydroxyethylene transition-state isostere of the LA peptide bond) and exhibits a K_i towards recombinant β -secretase produced in *E. coli* of $6.84 \times 10^{-8} \text{ M} \pm 2.72 \times 10^{-9} \text{ M}$. OM99-2 has the structure EVNL*AAEF (with "L*A" indicating the uncleavable hydroxyethylene transition-state isostere of the LA peptide bond) and exhibits a K_i towards recombinant β -secretase produced in *E. coli* of $9.58 \times 10^{-9} \text{ M} \pm 2.86 \times 10^{-10} \text{ M}$. OM99-1 and OM99-2, as well as related substances, are described in International Patent Publication WO0100665.

Currently, most drug discovery programs for Alzheimer's disease have targeted either acetylcholinesterase or the secretase proteins directly responsible for APP processing. While acetylcholinesterase inhibitors are marketed drugs for Alzheimer's disease, they have limited efficacy and do not have disease modifying properties. Secretase inhibitors, on the other hand, have been plagued either by mechanism-based toxicity (γ -secretase inhibitors) or by extreme difficulties in identifying small molecule inhibitors with appropriate pharmacokinetic properties to allow them to become drugs (BACE inhibitors). Identifying novel factors involved in APP processing would expand the range of targets for Alzheimer's disease treatments and therapy.

BRIEF SUMMARY OF THE INVENTION

The present invention provides methods for identifying modulators of KEAH6. The methods are particularly useful for identifying analytes that antagonize KEAH6's effect on processing of amyloid precursor protein to $A\beta$ peptide and thus useful for identifying analytes that can be used for treating Alzheimer disease.

Therefore, in one embodiment, the present invention provides a method for screening for analytes that antagonize processing of amyloid precursor protein (APP) to $A\beta$ peptide, comprising providing recombinant cells, which ectopically expresses KEAH6 and the APP; incubating the cells in a culture medium under conditions for expression of the KEAH6 and APP and which contains an analyte; removing the culture medium from the recombinant cells; and determining the amount of at least one processing product of APP selected from the group consisting of sAPP β and $A\beta$ peptide in the medium wherein a decrease in the amount of the processing product in the medium compared to the amount of the processing product in medium from recombinant cells incubated in medium without the analyte indicates that the analyte is an antagonist of the processing of the APP to $A\beta$ peptide.

In further aspects of the method, the recombinant cells each comprises a first nucleic acid that encodes KEAH6 operably linked to a first heterologous promoter and a second nucleic acid that encodes an APP operably linked to a second heterologous promoter. In preferred aspects of the present

invention, the APP is APP_{NFEV}. In preferred aspects, the method includes a control which comprises providing recombinant cells that ectopically express the APP but not the KEAH6.

The present invention further provides a method for screening for analytes that antagonize processing of amyloid precursor protein (APP) to amyloid β (A β) peptide, comprising providing recombinant cells, which ectopically express KEAH6 and a recombinant APP comprising APP fused to a transcription factor that when removed from the APP during processing of the APP produces an active transcription factor, and a reporter gene operably linked to a promoter inducible by the transcription factor; incubating the cells in a culture medium under conditions for expression of the KEAH6 and recombinant APP and which contains an analyte; and determining expression of the reporter gene wherein a decrease in expression of the reporter gene compared to expression of the reporter gene in recombinant cells in a culture medium without the analyte indicates that the analyte is an antagonist of the processing of the APP to A β peptide.

In further aspects of the method, the recombinant cells each comprises a first nucleic acid that encodes KEAH6 operably linked to a first heterologous promoter, a second nucleic acid that encodes the recombinant APP operably linked to a second heterologous promoter, and a third nucleic acid that encodes a reporter gene operably linked to promoter responsive to the transcription factor comprising the recombinant APP.

In light of the analytes that can be identified using the above methods, the present invention further provides a method for treating Alzheimer's disease in an individual which comprises providing to the individual an effective amount of an antagonist of KEAH6 activity.

Further still, the present invention provides a method for identifying an individual who has Alzheimer's disease or is at risk of developing Alzheimer's disease comprising obtaining a sample from the individual and measuring the amount of KEAH6 in the sample.

Further still, the present invention provides for the use of an antagonist of KEAH6 for the manufacture of a medicament for the treatment of Alzheimer's disease.

Further still, the present invention provides for the use of an antibody specific for KEAH6 for the manufacture of a medicament for the treatment of Alzheimer's disease.

Further still, the present invention provides a vaccine for preventing and/or treating Alzheimer's disease in a subject, comprising an antibody raised against an antigenic amount of KEAH6 wherein the antibody antagonizes the processing of APP to A β peptide.

The term "analyte" refers to a compound, chemical, agent, composition, antibody, peptide, aptamer, nucleic acid, or the like, which can modulate the activity of KEAH6.

The term "KEAH6" refers to KEAP1 related Alzheimer Heritability Chromosome 6 (Official Gene Designation KIAA1900), which is a gene from a human or another mammal having an open reading frame coding for a protein of 620 amino acids in length (SEQ ID NO:1) with an approximate molecular weight of about 70 kDa. The KEAH6 protein is predicted to have sequence

similarity and overall structure to KEAP1, an inhibitor of cellular detoxifications and anti-oxidant systems. The protein sequence is identical to the protein product of Genbank ID number NP_443136, from which the coding sequence, as reported in Genbank ID number NM_052904, was predicted. The term further includes mutants, variants, alleles, and polymorphs of KEAH6. Where appropriate, the term further includes fusion proteins comprising all or a portion of the amino acid sequence of KEAH6 fused to the amino acid sequence of a heterologous peptide or polypeptide, for example, hybrid immuoglobulins comprising the amino acid sequence of KEAH6 or KEAH6 fused at its C-terminus to the N-terminus of an immunoglobulin constant region amino acid sequence (*see*, for example, U.S. Patent No. 5,428,130 and related patents).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a nucleic sequence encoding the human KEAH6.

Figure 2 is the amino acid sequence of the human KEAH6.

Figure 3 is a graph showing the Relative expression of the metabolites expressed as a percent of the mean control non-silencing siRNA value of 100. KEAH6 $p < 0.05$ for EV40, EV42, and sAPP α and $p \approx 0.5$ for sAPP β .

Figure 4 shows the tissue distribution of KEAH6 mRNA in various human tissues.

Figure 5 shows that the gene encoding KEAH6 is located on chromosome 6q14-16.

Figure 6 is a dendograph showing the relationship of KEAH6 to KEAP1.

DETAILED DESCRIPTION OF THE INVENTION

The protein referred to herein as KEAH6 is a neuronal associated protein that the applicants have discovered to have a role in processing of amyloid precursor protein (APP) to amyloid β (A β) peptide. KEAH6 is predicted to have sequence similarity and overall structure to KEAP1, an inhibitor of cellular detoxification and anti-oxidant systems (Itoh, K., *Genes Dev.* 13(1):76-86 (1999)). Oxidative damage is generally accepted as occurring during aging and neurodegeneration. A defining characteristic of Alzheimer's disease (AD) is the deposition of aggregated plaques containing A β peptide in the brains of affected individuals. The applicant's discovery that KEAH6 has a role processing APP to A β peptide suggests that KEAH6 has a role in the progression of Alzheimer's disease in an individual. Therefore, in light of the applicants' discovery, identifying molecules which target activity or expression of KEAH6 would be expected to lead to treatments or therapies for Alzheimer's disease. Expression or activity of KEAH6 may also be useful as a diagnostic marker for identifying individuals who have Alzheimer's disease or are at risk of developing Alzheimer's disease.

The deposition of aggregated plaques containing amyloid β (A β) peptide in the brains of individuals affected with Alzheimer's disease is believed to involve the sequential cleavage of APP by two secretase-mediated cleavages to produce A β peptide. The first cleavage event is catalyzed by the

type I transmembrane aspartyl protease BACE1. BACE1 cleavage of APP at the BACE cleavage site (between amino acids 596 and 597) generates a 596 amino acid soluble N-terminal sAPP β fragment and a 99 amino acid C-terminal fragment (β CTF) designated C99. Further cleavage of C99 by γ -secretase (a multicomponent membrane complex consisting of at least presenilin, nicastrin, aph1, and pen2) releases the 40 or 42 amino acid A β peptide. An alternative, non-amyloidogenic pathway of APP cleavage is catalyzed by γ -secretase, which cleaves APP to produce a 613 amino acid soluble sAPP α N-terminal fragment and an 83 amino acid β CTF fragment designated C83. While ongoing drug discovery efforts have focused on identifying antagonists of BACE1 and γ -secretase mediated cleavage of APP, the complicated nature of Alzheimer's disease suggests that efficacious treatments and therapies for Alzheimer's disease might comprise other targets for modulating APP processing. KEAH6 of the present invention is another target for which modulators (in particular, antagonists) of are expected to provide efficacious treatments or therapies for Alzheimer's disease, either alone or in combination with one or more other modulators of APP processing, for example, antagonists selected from the group consisting of BACE1 and γ -secretase.

KEAH6 was identified by screening a siRNA library for siRNA that inhibited APP processing. As described in Example 1, a library of about 15,200 siRNA pools, each targeting a single gene, was transfected individually into recombinant cells ectopically expressing a recombinant APP (APP_{NFEV}). APP_{NFEV} has been described in U.S. Pub. Pat. Appln. No. 20030200555, comprises isoform 1-695 and has a HA, Myc, and FLAG sequences at the amino acid position 289, an optimized β -cleavage site comprising amino acids NFEV, and a K612V mutation. Metabolites of APP_{NFEV} produced during APP BACE1/ γ -secretase or α -secretase processing are sAPP β with NF at the C-terminus, EV40, and EV42 or sAPP α . EV40 and EV42 are unique A β 40-like and A β 42-like peptides that contain the glutamic acid and valine substitutions of APP_{NFEV} and sAPP β and sAPP α each contain the HA, FLAG, and myc sequences. sAPP β , sAPP α , EV40, and EV42 were detected by an immunodetection method that used antibodies that were specific for the various APP_{NFEV} metabolites. Expression levels were determined relative to a non-silencing siRNA control.

Following two rounds of screening, which consisted of a primary screen done with the entire library of siRNAs and secondary screening of about 1600 siRNAs performed in triplicate repeats, a siRNA designed to target KEAH6 RNA was found to consistently alter processing of APP to sAPP β , EV40, and EV42. The nucleic acid targeted by the siRNA has sequence identity to the human KEAH6, GenBank accession number NM_052904, from which the protein sequence NP_443136 was predicted and submitted (but unpublished) by Isogai, T. *et al.* The unpublished GenBank sequence in NM_052904 is identical to the derived cDNA sequence disclosed, but uncharacterized, in Isogai, T. *et al.*, EP 1 293 569 A2, SEQ ID NO: 324. Von der Kammer, H. *et al.*, found a gene coding for Ensadin-0581 that was homologous to a protein product (GenBank identification number Q96NJ5), which is identical to human KEAH6, WO 2004/003563. Ensadin-0581 was characterized as a gene found in specific brain regions of

Alzheimer's disease patients that could be used for diagnosis or prognostic assays, as a therapeutic, or to screen for modulators to treat Alzheimer's disease.

The nucleic acid sequence encoding the human KEAH6 (SEQ ID NO:1) is shown in Figure 1 and the amino acid sequence for the human KEAH6 (SEQ ID NO:2) is shown in Figure 2.

5 The mRNA encoding KEAH6 was found to be preferentially enriched in regions of the brain subject to Alzheimer's disease pathology (Example 2) and the gene encoding KEAH6 resides within chromosome 6 (Example 3), a genomic location that has been implicated to encode genes involved in late onset Alzheimer's disease.

10 In light of the applicants' discovery, KEAH6 or modified mutants or variants thereof is useful for identifying analytes which antagonize processing of APP to produce A β peptide. These analytes can be used to treat patients afflicted with Alzheimer's disease. KEAH6 can also be used to help diagnose Alzheimer's disease by assessing genetic variability within the locus. KEAH6 can be used alone or in combination with acetylcholinesterase inhibitors, NMDA receptor partial agonists, secretase inhibitors, amyloid-reactive antibodies, growth hormone secretagogues, and other treatments for
15 Alzheimer's disease.

The present invention provides methods for identifying KEAH6 modulators that modulate expression of KEAH6 by contacting KEAH6 with a substance that inhibits or stimulates KEAH6 expression and determining whether expression of KEAH6 polypeptide or nucleic acid molecules encoding an KEAH6 are modified. The present invention also provides methods for
20 identifying modulators that antagonize KEAH6's effect on processing APP to A β peptide or formation of A β -amyloid plaques in tissues where KEAH6 is localized or co-expressed. For example, KEAH6 protein can be expressed in cell lines that also express APP and the effect of the modulator on A β production is monitored using standard biochemical assays with A β -specific antibodies or by mass spectrophotometric techniques. Inhibitors for KEAH6 are identified by screening for a reduction in the release of A β peptide
25 which is dependent on the presence of KEAH6 protein for effect. Both small molecules and larger biomolecules that antagonize KEAH6-mediated processing of APP to A β peptide can be identified using such an assay. A method for identifying antagonists of KEAH6's effect on the processing APP to A β peptide includes the following method which is amenable to high throughput screening. In addition, the methods disclosed in U.S. Pub. Pat. Appln. No. 20030200555 can be adapted to use in assays for
30 identifying antagonists of KEAH6 activity.

A mammalian KEAH6 cDNA, encompassing the first through the last predicted codon contiguously, is amplified from brain total RNA with sequence-specific primers by reverse-transcription polymerase chain reaction (RT-PCR). The amplified sequence is cloned into pcDNA3.zeo or other appropriate mammalian expression vector. Fidelity of the sequence and the ability of the plasmid to
35 encode full-length KEAH6 is validated by DNA sequencing of the KEAH6 plasmid (pcDNA_KEAH6).

Commercially available mammalian expression vectors which are suitable for recombinant KEAH6 expression include, but are not limited to, pcDNA3.neo (Invitrogen, Carlsbad, CA), pcDNA3.1 (Invitrogen, Carlsbad, CA), pcDNA3.1/Myc-His (Invitrogen), pCI-neo (Promega, Madison, WI), pLITMUS28, pLITMUS29, pLITMUS38 and pLITMUS39 (New England Biolabs, Beverly, MA),
 5 pcDNAI, pcDNAIamp (Invitrogen), pcDNA3 (Invitrogen), pMC1neo (Stratagene, La Jolla, CA), pXT1 (Stratagene), pSG5 (Stratagene), EBO-pSV2-neo (ATCC 37593) pBPV-1(8-2) (ATCC 37110), pdBPV-MMTneo (342-12) (ATCC 37224), pRSVgpt (ATCC 37199), pRSVneo (ATCC 37198), pSV2-dhfr (ATCC 37146), pUCTag (ATCC 37460), lZD35 (ATCC 37565), pMC1neo (Stratagene), pcDNA3.1, pCR3.1 (Invitrogen, San Diego, Calif.), EBO-pSV2-neo (ATCC 37593), pCI-neo (Promega), pTRE
 10 (Clontech, Palo Alto, Calif.), pV1Jneo, pIRESneo (Clontech, Palo Alto, Calif.), pCEP4 (Invitrogen), pSC11, and pSV2-dhfr (ATCC 37146). The choice of vector will depend upon the cell type in which it is desired to express the KEAH6, as well as on the level of expression desired, cotransfection with expression vectors encoding APP_{NFEV}, and the like.

Cells transfected with plasmid vector comprising APP_{NFEV}, for example the
 15 HEK293T/APP_{NFEV} cells used to detect KEAH6 activity in the siRNA screening experiment described in Example 1, are used as described in Example 1 with the following modifications. Cells are either cotransfected with a plasmid expression vector comprising APP_{NFEV} operably linked to a heterologous promoter and a plasmid expression vector comprising the KEAH6 operably linked to a heterologous promoter or the HEK293T/APP_{NFEV} cells described in Example 1 and U.S. Pat. Appln.
 20 20030200555 are transfected with a plasmid expression vector comprising the KEAH6 operably linked to a heterologous promoter. The promoter comprising the plasmid expression vector can be a constitutive promoter or an inducible promoter. Preferably, the assay includes a negative control comprising the expression vector without the KEAH6.

After the cells have been transfected, the transfected or cotransfected cells are incubated
 25 with an analyte being tested for ability to antagonize KEAH6's effect on processing of APP to A β peptide. The analyte is assessed for an effect on the KEAH6 transfected or cotransfected cells that is minimal or absent in the negative control cells. In general, the analyte is added to the cell medium the day after the transfection and the cells incubated for one to 24 hours with the analyte. In particular embodiments, the analyte is serially diluted and each dilution provided to a culture of the transfected or
 30 cotransfected cells. After the cells have been incubated with the analyte, the medium is removed from the cells and assayed for secreted sAPP α , sAPP β , EV40, and EV42 as described in Examples 1 and 5. Briefly, the antibodies specific for each of the metabolites is used to detect the metabolites in the medium. Preferably, the cells are assessed for viability.

Analytes that alter the secretion of one or more of EV40, EV42, sAPP α , or sAPP β in the
 35 presence of KEAH6 protein are considered to be modulators of KEAH6 and potentially useful as therapeutic agents for KEAH6-related diseases. Direct inhibition or modulation of KEAH6 can be

confirmed using binding assays using the full-length KEAH6, extracellular or intracellular domain thereof or a KEAH6 fusion proteins comprising the intracellular or extracellular domains coupled to a C-terminal FLAG, or other, epitopes. A cell-free binding assay using full-length KEAH6, extracellular or intracellular domain thereof or a KEAH6 fusion proteins or membranes containing the KEAH6
5 integrated therein and labeled-analyte can be performed and the amount of labeled analyte bound to the KEAH6 determined.

The present invention further provides a method for measuring the ability of an analyte to modulate the level of KEAH6 mRNA or protein in a cell. In this method, a cell that expresses KEAH6 is contacted with a candidate compound and the amount of KEAH6 mRNA or protein in the cell is
10 determined. This determination of KEAH6 levels may be made using any of the above-described immunoassays or techniques disclosed herein. The cell can be any KEAH6 expressing cell such as cell transfected with an expression vector comprising KEAH6 operably linked to its native promoter or a cell taken from a brain tissue biopsy from a patient.

The present invention further provides a method of determining whether an individual
15 has a KEAH6-associated disorder or a predisposition for a KEAH6-associated disorder. The method includes providing a tissue or serum sample from an individual and measuring the amount of KEAH6 in the tissue sample. The amount of KEAH6 in the sample is then compared to the amount of KEAH6 in a control sample. An alteration in the amount of KEAH6 in the sample relative to the amount of KEAH6 in the control sample indicates the subject has a KEAH6-associated disorder. A control sample is
20 preferably taken from a matched individual, that is, an individual of similar age, sex, or other general condition but who is not suspected of having a KEAH6 related disorder. In another aspect, the control sample may be taken from the subject at a time when the subject is not suspected of having a condition or disorder associated with abnormal expression of KEAH6.

Other methods for identifying inhibitors of KEAH6 can include blocking the interaction
25 between KEAH6 and the enzymes involved in APP processing or trafficking using standard methodologies for analyzing protein-protein interaction such as fluorescence energy transfer or scintillation proximity assay. Surface Plasmon Resonance can be used to identify molecules that physically interact with purified or recombinant KEAH6.

In accordance with yet another embodiment of the present invention, there are provided
30 antibodies having specific affinity for the KEAH6 or epitope thereof. The term "antibodies" is intended to be a generic term which includes polyclonal antibodies, monoclonal antibodies, Fab fragments, single V_H chain antibodies such as those derived from a library of camel or llama antibodies or camelized antibodies (Nuttall *et al.*, Curr. Pharm. Biotechnol. 1: 253-263 (2000); Muyldermans, J. Biotechnol. 74: 277-302 (2001)), and recombinant antibodies. The term "recombinant antibodies" is intended to be a
35 generic term which includes single polypeptide chains comprising the polypeptide sequence of a whole heavy chain antibody or only the amino terminal variable domain of the single heavy chain antibody (V_H

chain polypeptides) and single polypeptide chains comprising the variable light chain domain (V_L) linked to the variable heavy chain domain (V_H) to provide a single recombinant polypeptide comprising the Fv region of the antibody molecule (scFv polypeptides) (*see* Schmiedl *et al.*, *J. Immunol. Meth.* 242: 101-114 (2000); Schultz *et al.*, *Cancer Res.* 60: 6663-6669 (2000); Dübel *et al.*, *J. Immunol. Meth.* 178: 201-209 (1995); and in U.S. Patent No. 6,207,804 B1 to Huston *et al.*). Construction of recombinant single V_H chain or scFv polypeptides which are specific against an analyte can be obtained using currently available molecular techniques such as phage display (de Haard *et al.*, *J. Biol. Chem.* 274: 18218-18230 (1999); Saviranta *et al.*, *Bioconjugate* 9: 725-735 (1999); de Greeff *et al.*, *Infect. Immun.* 68: 3949-3955 (2000)) or polypeptide synthesis. In further embodiments, the recombinant antibodies include modifications such as polypeptides having particular amino acid residues or ligands or labels such as horseradish peroxidase, alkaline phosphatase, fluors, and the like. Further still embodiments include fusion polypeptides which comprise the above polypeptides fused to a second polypeptide such as a polypeptide comprising protein A or G.

The antibodies specific for KEAH6 can be produced by methods known in the art. For example, polyclonal and monoclonal antibodies can be produced by methods well known in the art, as described, for example, in Harlow and Lane, *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY (1988). The KEAH6 or fragments thereof can be used as immunogens for generating such antibodies. Alternatively, synthetic peptides can be prepared (using commercially available synthesizers) and used as immunogens. Amino acid sequences can be analyzed by methods well known in the art to determine whether they encode hydrophobic or hydrophilic domains of the corresponding polypeptide. Altered antibodies such as chimeric, humanized, CDR-grafted, or bifunctional antibodies can also be produced by methods well known in the art. Such antibodies can also be produced by hybridoma, chemical synthesis or recombinant methods described, for example, in Sambrook *et al.*, *supra.*, and Harlow and Lane, *supra.* Both anti-peptide and anti-fusion protein antibodies can be used (*see*, for example, Bahouth *et al.*, *Trends Pharmacol. Sci.* 12: 338 (1991); Ausubel *et al.*, *Current Protocols in Molecular Biology*, (John Wiley and Sons, N.Y. (1989)).

Antibodies so produced can be used for the immunoaffinity or affinity chromatography purification of KEAH6 or KEAH6/ligand or analyte complexes. The above referenced anti-KEAH6 antibodies can also be used to modulate the activity of KEAH6 in living animals, in humans, or in biological tissues isolated therefrom. Accordingly, contemplated herein are compositions comprising a carrier and an amount of an antibody having specificity for KEAH6 effective to block naturally occurring KEAH6 from binding its ligand or for effecting the processing of APP to A β peptide.

Therefore, in another aspect, the present invention further provides pharmaceutical compositions that antagonize KEAH6's effect on processing of APP to A β peptide. Such compositions include a KEAH6 nucleic acid, KEAH6 peptide, fusion protein comprising KEAH6 or fragment thereof coupled to a heterologous peptide or protein or fragment thereof, an antibody specific for KEAH6,

nucleic acid or protein aptamers, siRNA inhibitory to KEAH6 mRNA, analyte that is a KEAH6 antagonist, or combinations thereof, and a pharmaceutically acceptable carrier or diluent.

In a further still aspect, the present invention further provides a kit for in vitro diagnosis of disease by detection of KEAH6 in a biological sample from a patient. A kit for detecting KEAH6 preferably includes a primary antibody capable of binding to KEAH6; and a secondary antibody conjugated to a signal-producing label, the secondary antibody being capable of binding an epitope different from, i.e., spaced from, that to which the primary antibody binds. Such antibodies can be prepared by methods well-known in the art. This kit is most suitable for carrying out a two-antibody sandwich immunoassay, e.g., two-antibody sandwich ELISA.

Using derivatives of KEAH6 protein or cDNA, dominant negative forms of KEAH6 that could interfere with KEAH6-mediated APP processing to A β release can be identified. These derivatives could be used in gene therapy strategies or as protein-based therapies to block KEAH6 activity in afflicted patients. KEAH6 can be used to identify endogenous brain proteins that bind to KEAH6 using biochemical purification, genetic interaction, or other techniques common to those skilled in the art. These proteins or their derivatives can subsequently be used to inhibit KEAH6 activity and thus be used to treat Alzheimer's disease. Additionally, polymorphisms in the KEAH6 RNA or in the genomic DNA in and around KEAH6 could be used to diagnose patients at risk for Alzheimer's disease or to identify likely responders in clinical trials.

The following examples are intended to promote a further understanding of the present invention.

EXAMPLE 1

KEAH6 was identified in a screen of a siRNA library for modulators of APP processing.

A cell plate was prepared by plating HEK293T/APP_{NFEV} cells to the wells of a 384-well Corning PDL-coated assay plate at a density of about 2,000 cells per well in 40 μ L DMEM containing 10% fetal bovine serum (FBS) and antibiotics. The cell plate was incubated overnight at 37°C in 5% CO₂. HEK293T/APP_{NFEV} cells are a subclone of HEK293T cells stably transformed with the APP_{NFEV} plasmid described in U.S. Pub. Pat. Appl. No. 20030200555. In brief, APP_{NFEV} encodes human amyloid precursor protein (APP), isoform 1-695, modified at amino acid position 289 by an in-frame insertion of HA, Myc, and FLAG epitope amino acid sequences and at amino acid positions 595, 596, 597, and 598 by substitution of the amino acid sequence NFEV for the endogenous amino acid sequence KMDA sequence comprising the BACE1 cleavage site. Thus, the BACE cleavage site is a modified BACE1 cleavage site and BACE1 cleaves between amino acids F and E of NFEV. Maintenance of the plasmid within the subclone is achieved by culturing the cells in the presence of the antibiotic puromycin.

The next day, the cells in each of the wells of the cell plate were transfected with a siRNA library as follows. Oligofectamine™ (Invitrogen, Inc., Carlsbad, CA) was mixed with Opti-

MEM[®] (Invitrogen, Inc., Carlsbad, CA) at a ratio of 1 to 40 and 20 μ L of the mixture was added to each well of a different 384-well plate. To each well of the plate, 980 nL of a particular 10 μ M siRNA species was added and the plate incubated for ten minutes at room temperature. Afterwards, five μ L of each the siRNA/ Oligofectamine[™] / Opti-MEM[®] mixtures was added to a corresponding well in the cell plate
5 containing the HEK293/APP_{NFEV} cells. The cell plate was incubated for 24 hours at 37°C in 5% CO₂. Controls were provided which contained non-silencing siRNA or a siRNA that inhibited BACE1.

On the next day, for each of the wells of the cell plate, the siRNA and Oligofectamine[™] / Opti-MEM[®] mixture was removed and replaced with 70 μ L DMEM containing 10% FBS and MERCK compound A (see WO2003093252, Preparation of spirocyclic [1,2,5]thiadiazole derivatives as γ -
10 secretase inhibitors for treatment of Alzheimer's disease, Collins *et al.*), a γ -secretase inhibitor given at a final concentration equal to its IC₅₀ in cell-based enzyme assays. The cell plate was incubated for 24 hours at 37°C in 5% CO₂.

On the next day, for each of the wells of the cell plate, 64 μ L of the medium (conditioned medium) was removed and transferred to four 384-well REMP plates in 22, 22, 10, and 10
15 μ L aliquots for subsequent use in detecting sAPP α , EV42, EV40, sAPP β using AlphaScreen[™] (PerkinElmer, Wellesley, MA) detection technology. Viability of the cells was determined by adding 40 μ L 10% Alamar Blue (Serotec Inc., Raleigh, NC) in DMEM containing 10% FBS to each of the wells of the cell plate with the conditioned medium removed. The cell plate was then incubated at 37°C for two hours. The Acquest[™] (Molecular Devices Corporation, Sunnyvale, CA) plate reader was used to assay
20 fluorescence intensity (ex. 545 nm, em. 590 nm) as a means to confirm viability of the cells.

Assays for detecting and measuring sAPP β , EV42, EV40, and sAPP α were detected using antibodies as follows. In general, detection-specific volumes (8 or 0.5 μ L) were transferred to a 384-well, white, small-volume detection plate (Greiner Bio-One, Monroe, NC). In the case of the smaller volume, 7.5 μ L of assay medium was added for a final volume of eight μ L per well. One μ L of
25 antibody/donor bead mixture (see below) was dispensed into the solution, and one μ L antibody/acceptor bead mixture was added. Plates were incubated in the dark for 24 hours at 4°C. Then the plates were read using AlphaQuest[™] (PerkinElmer, Wellesley, MA) instrumentation. In all protocols, the plating medium was DMEM (Invitrogen, Carlsbad, CA; Cat. No. 21063-029); 10% FBS, the AlphaScreen[™] buffer was 50 mM HEPES, 150 mM NaCl, 0.1% BSA, 0.1% Tween-20, pH 7.5, and the AlphaScreen[™]
30 Protein A kit was used.

Anti-NF antibodies and anti-EV antibodies were prepared as taught in U.S. Pub. Pat. Appln. 20030200555. BACE1 cleaves between amino acids F and E of the NFEV cleavage site of APP_{NFEV} to produce an sAPP β peptide with NF at the C-terminus and an EV40 or EV42 peptide with amino acids EV at the N-terminus. Anti-NF antibodies bind the C-terminal neoepitope NF at the C-

terminus of the sAPP β peptide produced by BACE1 cleavage of the NFEV sequence of APP_{NFEV}.

Anti-EV antibodies bind the N-terminal neopeptide EV at the N-terminus of EV40 and EV42 produced by BACE1 cleavage of the NFEV sequence of APP_{NFEV}. Anti-Bio-G2-10 and anti-Bio-G2-11

antibodies are available from the Genetics Company, Zurich, Switzerland. Anti-Bio-G2-11 antibodies

5 bind the neopeptide generated by the γ -secretase cleavage of A β or EV peptides at the 42 amino acid position. Anti-Bio-G2-10 antibodies bind the neopeptide generated by the γ -secretase cleavage of A β or EV peptides at the 40 amino acid position. Anti-6E10 antibodies are commercially available from Signet Laboratories, Inc., Dedham, MA. Anti-6E10 antibodies bind the epitope within amino acids 1 to 17 of the N-terminal region of the A β and the EV40 and EV42 peptides and also binds sAPP α because the
10 same epitope resides in amino acids 597 to 614 of sAPP α . Bio-M2 anti-FLAG antibodies are available from Sigma-Aldrich, St. Louis, MO.

Detecting sAPP β . An AlphaScreenTM assay for detecting sAPP β -NF produced from cleavage of APP_{NFEV} at the BACE cleavage site was performed as follows. Conditioned medium for each well was diluted 32-fold into a final volume of eight μ L. As shown in Table 1, biotinylated-M2
15 anti-FLAG antibody, which binds the FLAG epitope of the APP_{NFEV}, was captured on streptavidin-coated donor beads by incubating a mixture of the antibody and the streptavidin coated beads for one hour at room temperature in AlphaScreenTM buffer. The amount of antibody was adjusted such that the final concentration of antibody in the detection reaction was 3 nM. Anti-NF antibody was similarly captured separately on protein-A acceptor beads in AlphaScreenTM buffer and used at a final
20 concentration of 1 nM (Table 1). The donor and acceptor beads were each used at final concentrations of 20 μ g/mL.

Table 1

Donor/Antibody Bead Mixture			Acceptor/Antibody Bead Mixture		
	Vol. (μ L)	Final Conc. in 50 μ L assay		Vol. (μ L)	Final Conc. in 50 μ L assay
Anti-Bio-Flag (16 μ M)	1	3 nM	NF-IgG (1.1 μ M)	5	1 nM
SA Coated Donor Beads (5 mg/mL)	23	20 μ g/mL	Protein A Acceptor Beads (5 mg/mL)	23	20 μ g/mL
Alpha Buffer	1131		Alpha Buffer	1127	
Final Vol.	1155		Final Vol.	1155	

Detecting EV42: Conditioned medium for each well was used neat (volume eight μ L). As shown in Table 2, anti-Bio-G2-11 antibody was captured on streptavidin-coated donor beads by
25 incubating a mixture of the antibody and the streptavidin coated beads for one hour at room temperature in AlphaScreenTM buffer. The amount of antibody was adjusted such that the final concentration of antibody in the detection reaction was 20 nM. Anti-EV antibody was similarly captured separately on

protein-A acceptor beads in AlphaScreen™ buffer and used at a final concentration of 5 nM (Table 2). The donor and acceptor beads were used at final concentrations of 20 µg/mL.

Table 2

Donor/Antibody Bead Mixture			Acceptor/Antibody Bead Mixture		
	Vol. (µL)	Final Conc. in 50 µL assay		Vol. (µL)	Final Conc. in 50 µL assay
Anti-Bio-G2-11 (8.27 µM)	14	20 nM	EV-IgG (1.27 µM)	23	5 nM
SA Coated Donor Beads (5 mg/mL)	23	20 µg/mL	Protein A Acceptor Beads (5 mg/mL)	23	20 µg/mL
Alpha Buffer	1118		Alpha Buffer	1109	
Final Vol.	1155		Final Vol.	1155	

- 5 Detecting EV40. Conditioned medium for each well was diluted four-fold into a final volume eight µL. As shown in Table 3, anti-Bio-G2-10 antibody was captured on streptavidin-coated donor beads by incubating a mixture of the antibody and the streptavidin coated beads for one hour at room temperature in AlphaScreen™ buffer. The amount of antibody was adjusted such that the final concentration of antibody in the detection reaction was 20 nM. Anti-EV antibody was similarly captured
- 10 separately on protein-A acceptor beads in AlphaScreen™ buffer and used at a final concentration of 5 nM. The donor and acceptor beads were used at final concentrations of 20 µg/mL.

Table 3

Donor/Antibody Bead Mixture			Acceptor/Antibody Bead Mixture		
	Vol. (µL)	Final Conc. in 50 µL assay		Vol. (µL)	Final Conc. in 50 µL assay
Anti-Bio-G2-10 (6.07 µM)	5	5 nM	EV-IgG (1.27 µM)	23	5 nM
SA Coated Donor Beads (5 mg/mL)	23	20 µg/mL	Protein A Acceptor Beads (5 mg/mL)	23	20 µg/mL
Alpha Buffer	1127		Alpha Buffer	1109	
Final Vol.	1155		Final Vol.	1155	

- 15 Detecting sAPPα: Conditioned medium for each well was diluted four-fold into a final volume eight µL. As shown in Table 4, Bio-M2 anti-FLAG antibody was captured on streptavidin-coated donor beads by incubating a mixture of the antibody and the streptavidin coated beads for one hour at room temperature in AlphaScreen™ buffer. Anti-6E10 antibody acceptor beads supplied by the manufacturer (Perkin-Elmer, Inc. makes the beads and conjugates antibody 6E10 to them; antibody 6E10 is made by Signet Laboratories, Inc.) were used at 30 µg/ml final concentration. The donor beads were
- 20 used at final concentrations of 20 µg/mL.

Table 4

Donor/Antibody Bead Mixture			Acceptor/Antibody Bead Mixture		
	Vol. (μL)	Final Conc. in 50 μL assay		Vol. (μL)	Final Conc. in 50 μL assay
Anti-Bio-Flag (16 μM)	1	5 nM	6E10-IgG (5 mg/mL)	34.65	30 μg/mL
SA Coated Donor Beads (5 mg/mL)	23	20 μg/mL			
Alpha Buffer	1131		Alpha Buffer	1120.35	
Final Vol.	1155		Final Vol.	1155	

About 15,200 single replicate pools of siRNAs were tested for modulation of sAPP β , sAPP α , EV40 and EV42 by the ALPHASCREEN immunodetection method as described above. Based on the profile from this primary screen, 1,622 siRNA were chosen for an additional round of screening in triplicate. siRNAs were defined as “secretase-like” if a significant decrease in sAPP β , EV40 and EV42 was detected as well as either no change or an increase in sAPP α .

A siRNA was identified which inhibited an mRNA having a nucleotide sequence encoding a protein which had 100% identity to the nucleotide sequence encoding KEAH6. Compared to control non-silencing siRNAs (set to 100%), KEAH6 siRNA pool significantly decreased EV40 (71.9%), EV42 (62.1%), while increasing sAPP α (303.8%), and decreasing sAPP β (87.9%).

The results are shown schematically in Figure 3 and show that KEAH6 has a role in APP processing, in particular, the cleavage of APP at the BACE cleavage site, an event necessary in the processing of APP to A β peptide. A β peptide is a defining characteristic of Alzheimer's disease. Because of its role APP processing, KEAH6 appears to have a role in the establishment or progression of Alzheimer's disease.

EXAMPLE 2

Because KEAH6 appeared to have a role in APP processing to A β peptide and thus, a role in progression of Alzheimer's disease, expression of KEAH6 was examined in a variety of tissues to determine whether KEAH6 was expressed in the brain.

A proprietary database, the TGI Body Atlas, was used to show that the results of a microarray analysis of the expression of a majority of characterized genes, including KEAH6, in the human genome in a panel of different tissues. KEAH6 mRNA was found to be expressed predominantly in the brain and within cortical structures such as the temporal lobe, entorhinal cortex, and prefrontal cortex, all of which are subjected to amyloid A β deposition and Alzheimer pathology. The results are summarized in Figure 4.

The results strengthen the conclusion of the Example 1 that KEAH6 has a role in APP processing and thus, a role in the establishment or progression of Alzheimer's disease.

EXAMPLE 3

This example shows that KEAH6 is located within a region of the human genome known to be implicated in late onset of Alzheimer's disease, which further strengthens the conclusion that

KEAH6 has a role in the progression of Alzheimer's disease.

Several published population studies have defined genomic locations that influence an individual's propensity to develop Alzheimer's disease. Such studies are able to define particular genomic regions thought to harbor loci that when present or absent, alter an individual's chances of developing Alzheimer's disease. The presence of such loci within or near a gene's genomic location is thought to be a strong indicator of that particular gene's potential influence on disease onset or progression. Pericak-Vance, M.A. *et al.*, JAMA 278(15): 1237-1241 (1997), Curtis, D., *et al.*, Ann. Hum. Genet. 65 (Pt.5): 473-481 (2001) and Kehoe, P., *et al.*, Am. J. Hum. Gen. 8: 237-245 (1999) provided evidence suggesting that an Alzheimer's disease locus independent of the APOE genotype is located on chromosome 6 at or close to locus 6q14-16

Figure 5 shows the location of KEAH6 on chromosome 6 at q16.1 relative to the genomic area shown to have linkage to Alzheimer's disease in the above studies. KEAH6's close proximity to the linkage sites identified as being linked to risk for late-onset Alzheimer's disease further supports the conclusion that KEAH6 is involved in the establishment or progression of Alzheimer's disease. This possibility is supported by the observation that APOE, the only unequivocal genetic risk factor for Alzheimer's disease, gave a similar logarithm of the odds (LOD) score to the chromosome 6 locus in the Kehoe study (APOE LOD = 1.79, chromosome 6 LOD = 1.40 in APOE positive subjects, Kehoe, P., *et al.*, Am. J. Hum. Gen. 8: 237-245 (1999). Thus, these studies were capable of detecting genuine risk factors for Alzheimer's disease, supporting the possibility that KEAH6, a gene with the ability to modify A β secretion, plays a role in the initiation or progression of Alzheimer's disease.

EXAMPLE 4

As shown in Figure 6, KEAH6, having a BTB and five Kelch domains, shares sequence and domain homology to KEAP1, an inhibitor of cellular detoxification and anti-oxidant systems, Itoh, K., *et al.*, Genes Dev. 13(1): 76-86 (1999). Overall, KEAH6 protein is 25% identical and 44% similar to KEAP1 protein (Figure 6). KEAP1 functions to sequester NRF2 in the cytoplasm and induce its proteasomal degradation under normal conditions (reviewed by Kobayashi M, and Yamamoto M, Antioxid Redox Signal. 7:385-94 (2005)). The Kelch domains in the C-terminus of KEAP1 physically interact with NRF2 to accomplish this sequestration. Following stresses such as oxidants, the KEAP1-NRF2 interaction is disrupted and NRF2 translocates to the nucleus to induce the expression of genes involved in detoxification and antioxidation. As oxidative damage is generally accepted as occurring during aging and neurodegeneration, this similarity in overall structure between KEAH6 and KEAP1 supports the possibility that KEAH6 plays a role in the initiation or progression of Alzheimer's disease.

EXAMPLE 5

The results of Examples 1-4 have shown that the KEAH6 has a role in the establishment or progression of Alzheimer's disease. The results suggest that analytes that antagonize KEAH6 activity will be useful for the treatment or therapy of Alzheimer's disease. Therefore, there is a need for assays for identifying analytes that antagonize KEAH6 activity, for example, inhibit binding of KEAH6 to its natural ligand or to BACE1. The following is an assay that can be used to identify analytes that antagonize KEAH6 activity.

HEK293T/APP_{NFEV} cells are transfected with a plasmid encoding the human KEAH6 or a homolog of the human KEAH6, for example, the primate, rodent, or other mammalian KEAH6, using a standard transfection protocols to produce HEK293T/APP_{NFEV}/KEAH6 cells. For example, HEK293T/APP_{NFEV} are plated into a 96-well plate at about 8000 cells per well in 80 μ L DMEM containing 10%FBS and antibiotics and the cell plate incubated at 37°C at 5% CO₂ overnight.

On the next day, a mixture of 600 μ L OligofectamineTM and 3000 μ L Opti-MEM[®] is made and incubated at room temperature for five minutes. Next, 23 μ L Opti-MEM is added to each well of a 96-well mixing plate. 50 ng pcDNA_KEAH6 and empty control vector (in 1 μ L volume) are added into adjacent wells of the mixing plate in an alternating fashion. The mixing plate is incubated at room temperature for five minutes. Next, 6 μ L of the Oligofectamine mixture is added to each of the wells of the mixing plate and the mixing plate incubated at room temperature for five minutes. After five minutes, 20 μ L of the plasmid/Oligofectamine mixture is added to the corresponding well in the plate of HEK293/APP_{NFEV} cells plated in the cell plate and the plates incubated overnight at 37°C in 5% CO₂.

The next day, the medium is removed from each well and replaced with 100 μ L DMEM containing 10% FBS. Analytes being assayed for the ability to antagonize KEAH6-mediated activation of A β secretion are added to each well individually. The analytes are assessed for an effect on the APP processing to A β peptide in KEAH6 transfected cells that is either minimal or absent in cells transfected with the vector-alone as follows. The cells are incubated at 37°C at 5% CO₂ overnight.

The next day, conditioned media is collected the amount of sAPP β , EV42, EV40, and sAPP α in the conditioned media is determined as described in Example 1. Analytes that effect a decrease in the amounts of sAPP β , EV42, and EV40 and either an increase or no change in the amount of sAPP α are antagonists of KEAH6. Viability of the cells is determined as in Example 1.

EXAMPLE 6

Analytes that alter secretion of EV40, EV42, sAPP α , or sAPP β only, or more, in the presence of KEAH6 are considered to be modulators of KEAH6 and potential therapeutic agents for treating KEAH6-related diseases. The following is an assay that can be used to confirm direct inhibition or modulation of KEAH6.

To confirm direct inhibition or modulation of KEAH6, KEAH6 intracellular or extracellular domains are subcloned into expression plasmid vectors such that a fusion protein with C-terminal FLAG epitopes are encoded. These fusion proteins are purified by affinity chromatography, according to manufacturer's instructions, using an ANTI-FLAG M2 agarose resin. KEAH6 fusion proteins are eluted from the ANTI-FLAG column by the addition of FLAG peptide (Asp-Tyr-Lys-Asp-Asp-Asp-Lys) (Sigma Aldrich, St. Louis, MO) resuspended in TBS (50 mM Tris HCl pH 7.4, 150 mM NaCl) to a final concentration of 100 µg/ml. Fractions from the column are collected and concentrations of the fusion proteins determined by A280.

A PD-10 column (Amersham, Boston, MA) is used to buffer exchange all eluted fractions containing the KEAH6-fusion proteins and simultaneously remove excess FLAG peptide. The FLAG-KEAH6 fusion proteins are then conjugated to the S series CM5 chip surface (Biacore™ International SA, Uppsala, Sweden) using amine coupling as directed by the manufacturer. A pH scouting protocol is followed to determine the optimal pH conditions for immobilization. Immobilization is conducted at an empirically determined temperature in PBS, pH 7.4, or another similar buffer following a standard Biacore™ immobilization protocol. The reference spot on the CM5 chip (a non-immobilized surface) serves as background. A third spot on the CM5 chip is conjugated with bovine serum albumin in a similar fashion to serve as a specificity control. Interaction of the putative KEAH6 modulating analyte identified in the assay of Example 5 at various concentrations and KEAH6 are analyzed using the compound characterization wizard on the Biacore™ S51. Binding experiments are completed at 30°C using 50 mM Tris pH 7, 200 uM MnCl₂ or MgCl₂ (+ 5% DMSO) or a similar buffer as the running buffer. Prior to each characterization, the instrument is equilibrated three times with assay buffer. Default instructions for characterization are a contact time of 60 seconds, sample injection of 180 seconds and a baseline stabilization of 30 seconds. All solutions are added at a rate of 30 µL/min. Using the BiaEvaluation software (Biacore™ International AB, Uppsala, Sweden), each set of sensorgrams derived from the ligand flowing through the KEAH6-conjugated sensor chip is evaluated and, if binding is observed, an affinity constant determined.

EXAMPLE 7

This example describes a method for making polyclonal antibodies specific for the KEAH6 or particular peptide fragments or epitope thereof.

The KEAH6 is produced as described in Example 1 or a peptide fragment comprising a particular amino acid sequence of KEAH6 is synthesized and coupled to a carrier such as BSA or KLH. Antibodies are generated in New Zealand white rabbits over a 10-week period. The KEAH6 or peptide fragment or epitope is emulsified by mixing with an equal volume of Freund's complete adjuvant and injected into three subcutaneous dorsal sites for a total of about 0.1 mg KEAH6 per immunization. A booster containing about 0.1 mg KEAH6 or peptide fragment emulsified in an equal volume of Freund's incomplete adjuvant is administered subcutaneously two weeks later. Animals are bled from the articular

artery. The blood is allowed to clot and the serum collected by centrifugation. The serum is stored at -20°C.

For purification, the KEAH6 is immobilized on an activated support. Antisera is passed through the sera column and then washed. Specific antibodies are eluted via a pH gradient, collected, and stored in a borate buffer (0.125M total borate) at -0.25 mg/mL. The anti-KEAH6 antibody titers are determined using ELISA methodology with free cS1P5 receptor bound in solid phase (1 pg/well). Detection is obtained using biotinylated anti-rabbit IgG, HRP-SA conjugate, and ABTS.

EXAMPLE 8

This example describes a method for making monoclonal antibodies specific for KEAH6.

BALB/c mice are immunized with an initial injection of about 1 µg of purified KEAH6 per mouse mixed 1:1 with Freund's complete adjuvant. After two weeks, a booster injection of about 1 µg of the antigen is injected into each mouse intravenously without adjuvant. Three days after the booster injection serum from each of the mice is checked for antibodies specific for KEAH6.

The spleens are removed from mice positive for antibodies specific for the KEAH6 and washed three times with serum-free DMEM and placed in a sterile Petri dish containing about 20 mL of DMEM containing 20% fetal bovine serum, 1 mM pyruvate, 100 units penicillin, and 100 units streptomycin. The cells are released by perfusion with a 23 gauge needle. Afterwards, the cells are pelleted by low-speed centrifugation and the cell pellet is resuspended in 5 mL 0.17 M ammonium chloride and placed on ice for several minutes. Then 5 mL of 20% bovine fetal serum is added and the cells pelleted by low-speed centrifugation. The cells are then resuspended in 10 mL DMEM and mixed with mid-log phase myeloma cells in serum-free DMEM to give a ratio of 3:1. The cell mixture is pelleted by low-speed centrifugation, the supernatant fraction removed, and the pellet allowed to stand for 5 minutes. Next, over a period of 1 minute, 1 mL of 50% polyethylene glycol (PEG) in 0.01 M HEPES, pH 8.1, at 37°C is added. After 1 minute incubation at 37°C, 1 mL of DMEM is added for a period of another 1 minute, then a third addition of DMEM is added for a further period of 1 minute. Finally, 10 mL of DMEM is added over a period of 2 minutes. Afterwards, the cells are pelleted by low-speed centrifugation and the pellet resuspended in DMEM containing 20% fetal bovine serum, 0.016 mM thymidine, 0.1 hypoxanthine, 0.5 µM aminopterin, and 10% hybridoma cloning factor (HAT medium). The cells are then plated into 96-well plates.

After 3, 5, and 7 days, half the medium in the plates is removed and replaced with fresh HAT medium. After 11 days, the hybridoma cell supernatant is screened by an ELISA assay. In this assay, 96-well plates are coated with the KEAH6. One hundred µL of supernatant from each well is added to a corresponding well on a screening plate and incubated for 1 hour at room temperature. After incubation, each well is washed three times with water and 100 µL of a horseradish peroxidase conjugate of goat anti-mouse IgG (H+L), A, M (1:1,500 dilution) is added to each well and incubated for 1 hour at

room temperature. Afterwards, the wells are washed three times with water and the substrate OPD/hydrogen peroxide is added and the reaction is allowed to proceed for about 15 minutes at room temperature. Then 100 μ L of 1 M HCl is added to stop the reaction and the absorbance of the wells is measured at 490 nm. Cultures that have an absorbance greater than the control wells are removed to two
5 cm^2 culture dishes, with the addition of normal mouse spleen cells in HAT medium. After a further three days, the cultures are re-screened as above and those that are positive are cloned by limiting dilution. The cells in each two cm^2 culture dish are counted and the cell concentration adjusted to 1×10^5 cells per mL. The cells are diluted in complete medium and normal mouse spleen cells are added. The cells are plated in 96-well plates for each dilution. After 10 days, the cells are screened for growth.
10 The growth positive wells are screened for antibody production; those testing positive are expanded to 2 cm^2 cultures and provided with normal mouse spleen cells. This cloning procedure is repeated until stable antibody producing hybridomas are obtained. The stable hybridomas are progressively expanded to larger culture dishes to provide stocks of the cells.

Production of ascites fluid is performed by injecting intraperitoneally 0.5 mL of pristane
15 into female mice to prime the mice for ascites production. After 10 to 60 days, 4.5×10^6 cells are injected intraperitoneally into each mouse and ascites fluid is harvested between 7 and 14 days later.

While the present invention is described herein with reference to illustrated embodiments, it should be understood that the invention is not limited hereto. Those having ordinary skill in the art and access to the teachings herein will recognize additional modifications and
20 embodiments within the scope thereof. Therefore, the present invention is limited only by the claims attached herein.

WHAT IS CLAIMED:

1. A method for screening for analytes that antagonize processing of amyloid precursor protein (APP) to A β peptide, comprising:
 - (a) providing recombinant cells, which ectopically expresses KEAH6 and the APP;
 - (b) incubating the cells in a culture medium under conditions for expression of the KEAH6 and APP and which contains an analyte;
 - (c) removing the culture medium from the recombinant cells; and
 - (d) determining the amount of at least one processing product of APP selected from the group consisting of sAPP β and A β peptide in the medium wherein a decrease in the amount of the processing product in the medium compared to the amount of the processing product in medium from recombinant cells incubated in medium without the analyte indicates that the analyte is an antagonist of the processing of the APP to A β peptide.
2. The method of Claim 1 wherein the recombinant cells each comprises a first nucleic acid that encodes KEAH6 operably linked to a first heterologous promoter and a second nucleic acid that encodes an APP operably linked to a second heterologous promoter.
3. The method of Claim 2 wherein the APP is APP_{NFEV}.
4. The method of Claim 1 wherein a control is provided which comprises providing recombinant cells which ectopically express the APP but not the KEAH6.
5. A method for screening for analytes that antagonize processing of amyloid precursor protein (APP) to amyloid β (A β) peptide, comprising:
 - (a) providing recombinant cells, which ectopically express KEAH6 and a recombinant APP comprising APP fused to a transcription factor that when removed from the APP during processing of the APP produces an active transcription factor, and a reporter gene operably linked to a promoter inducible by the transcription factor;
 - (b) incubating the cells in a culture medium under conditions for expression of the KEAH6 and recombinant APP and which contains an analyte; and
 - (c) determining expression of the reporter gene wherein a decrease in expression of the reporter gene compared to expression of the reporter gene in recombinant cells in a culture medium without the analyte indicates that the analyte is an antagonist of the processing of the APP to A β peptide.
6. A method for treating Alzheimer's disease in an individual comprising providing to the individual an effective amount of an antagonist of KEAH6 activity.

7. A method for identifying an individual who has Alzheimer's disease or is at risk of developing Alzheimer's disease comprising obtaining a sample from the individual and measuring the amount of KEAH6 in the sample.

5

8. The use of an antagonist of KEAH6 for the manufacture of a medicament for the treatment of Alzheimer's disease.

9. The use of an antibody specific for KEAH6 for the manufacture of a medicament
10 for the treatment of Alzheimer's disease.

10. A vaccine for preventing and/or treating Alzheimer's disease in a subject, comprising an antibody raised against an antigenic amount of KEAH6 wherein the antibody antagonizes the processing of APP to A β peptide.

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(SEQ ID NO:1)

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FIG. 1

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(SEQ ID NO:2)

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FIG.2

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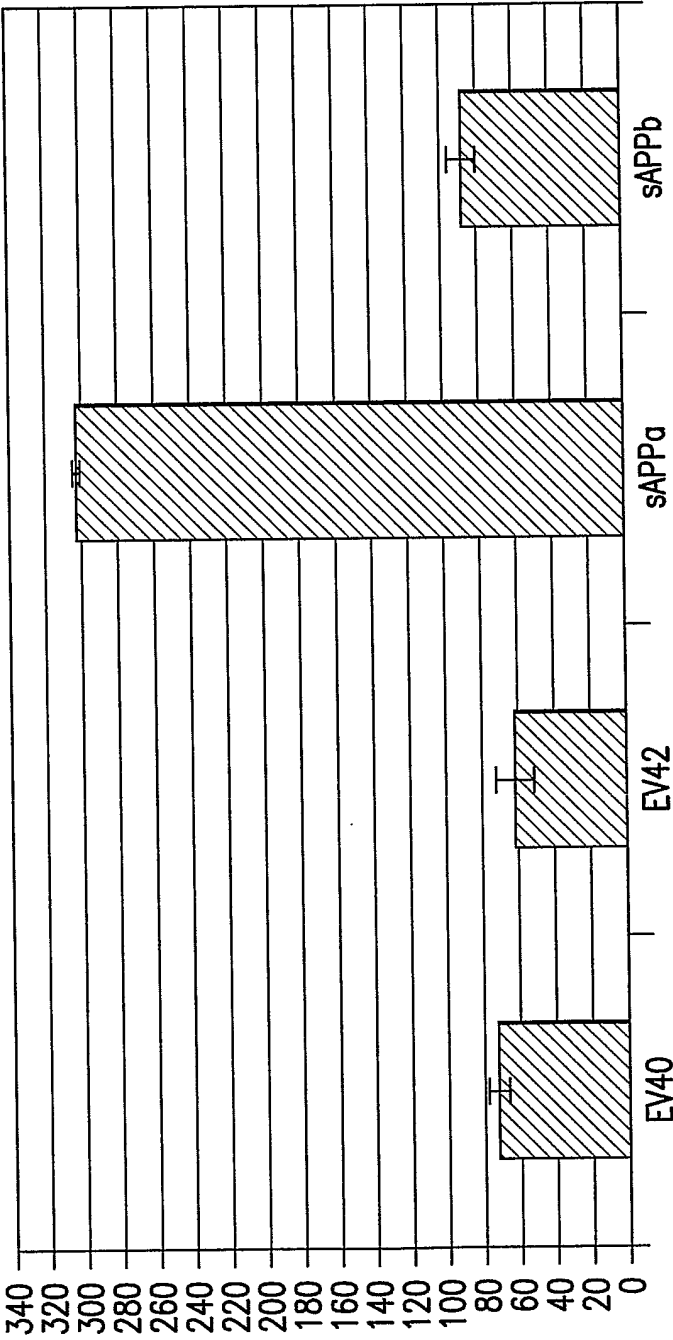


FIG. 3

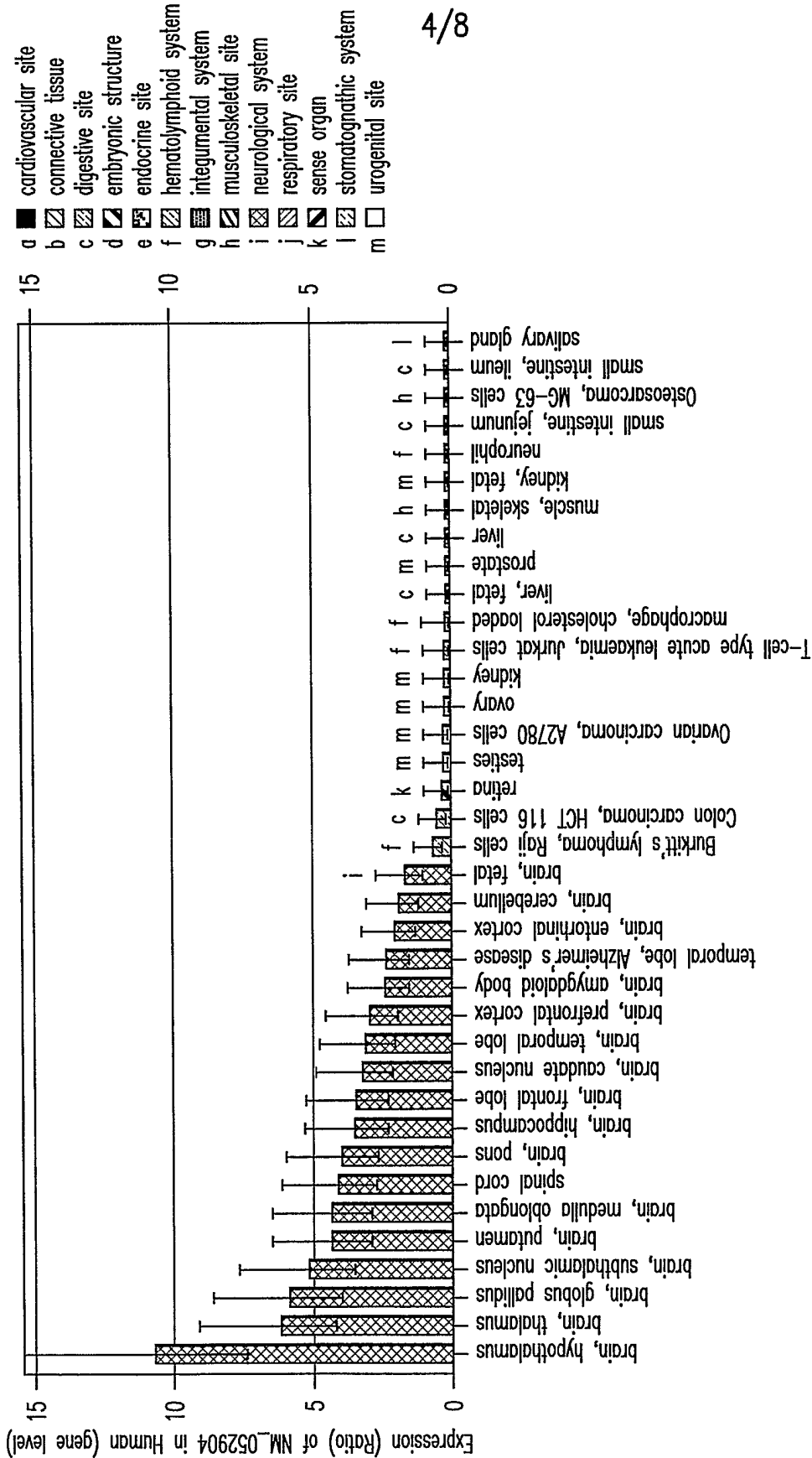


FIG.4A

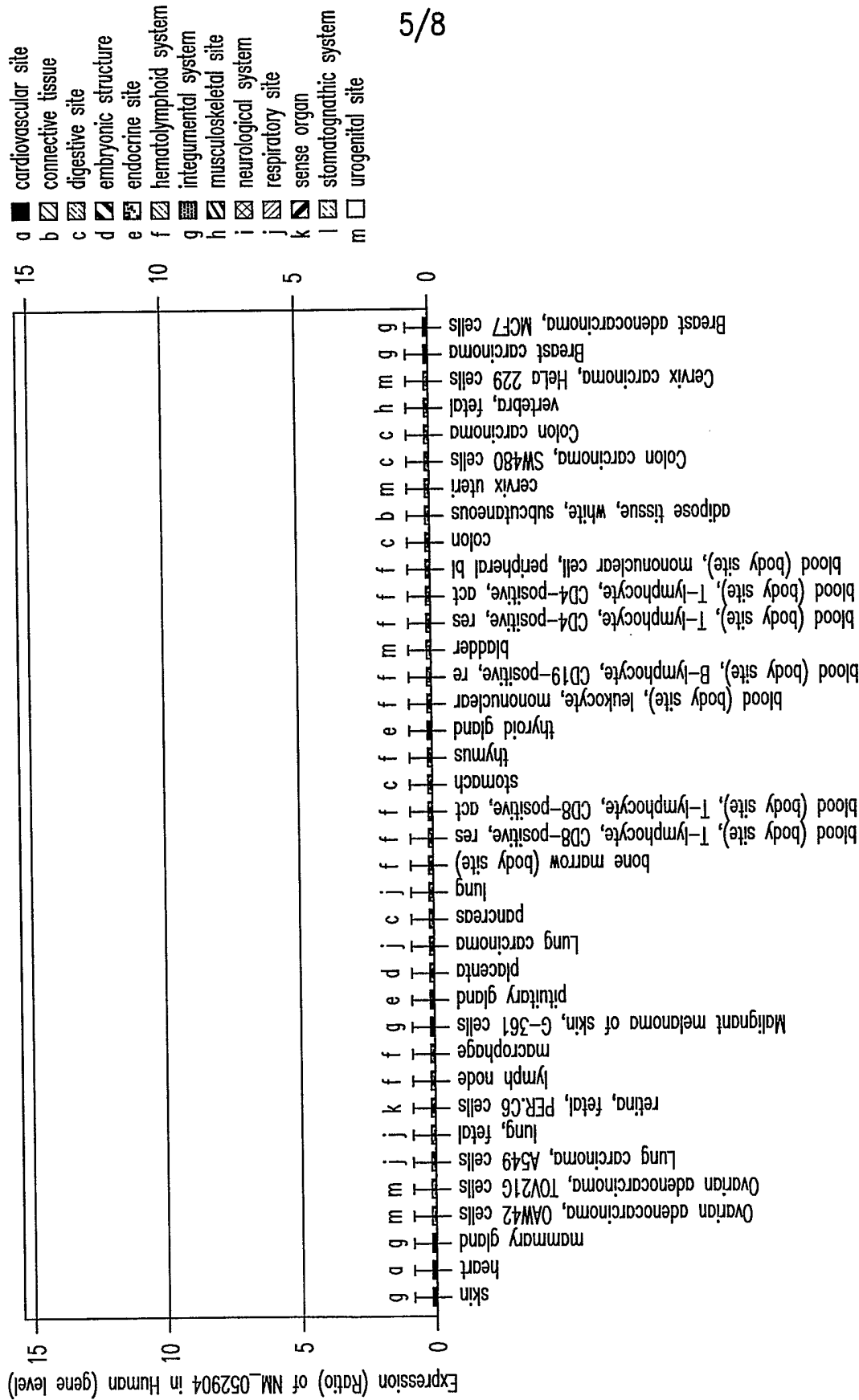


FIG.4B

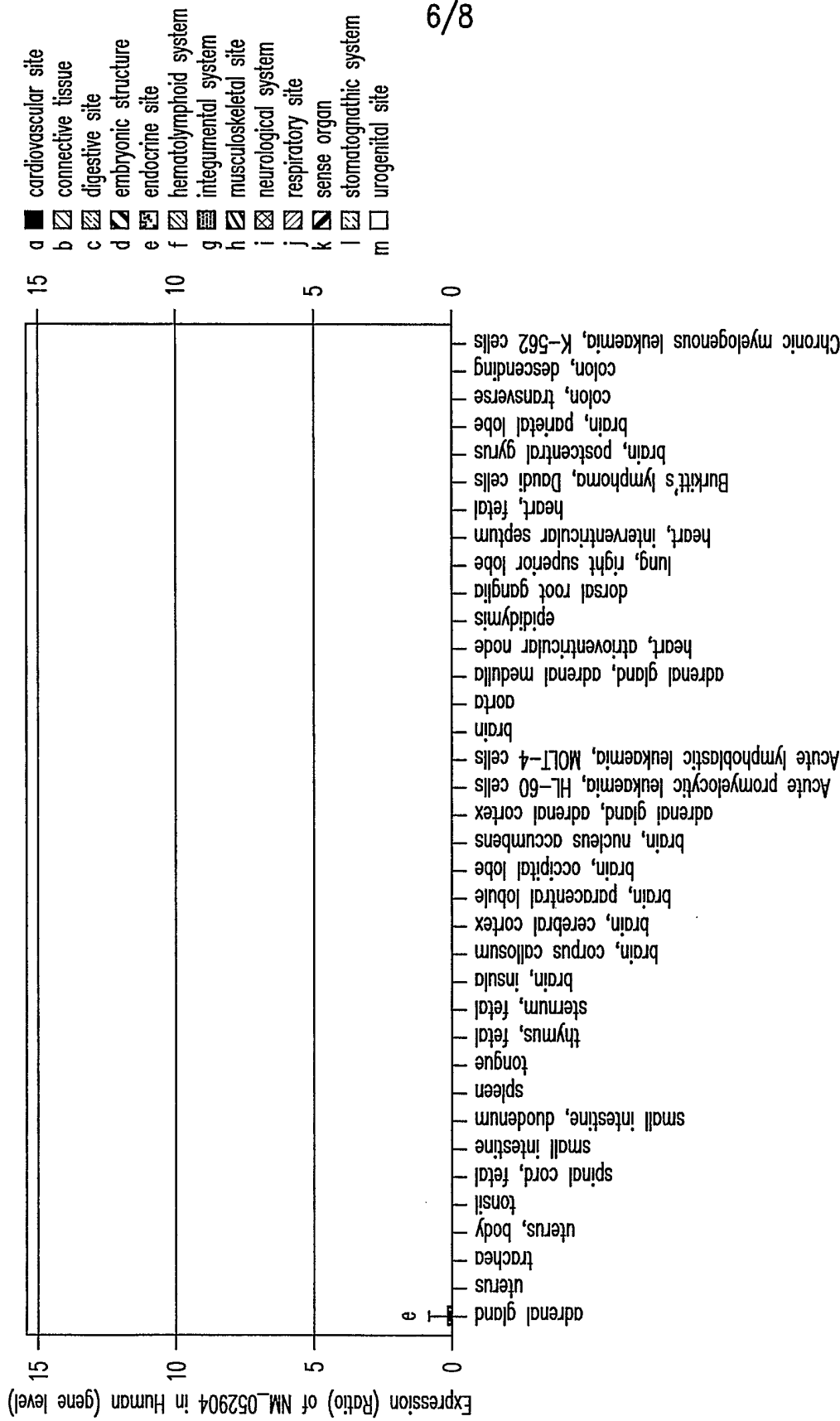


FIG.4C

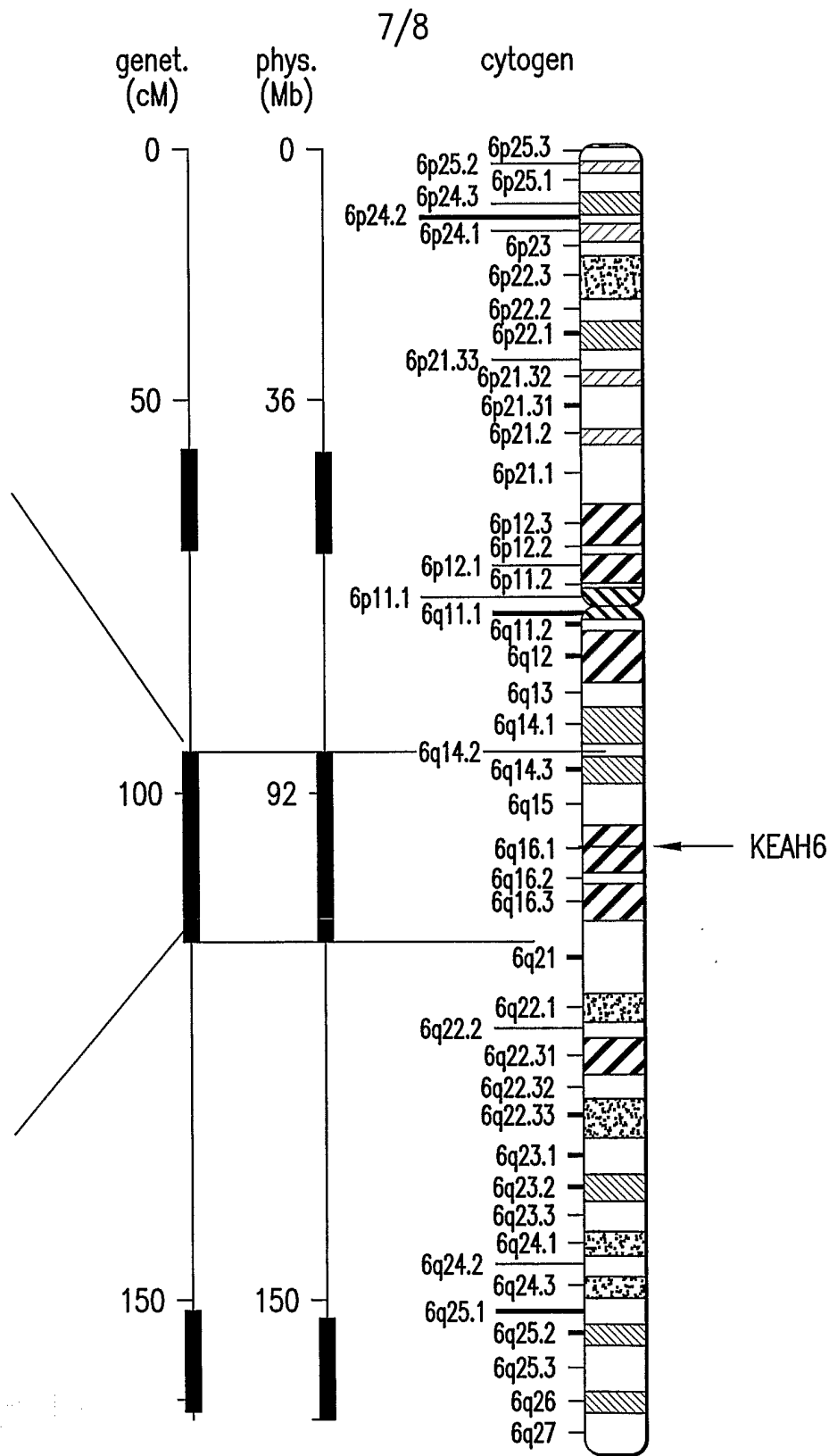
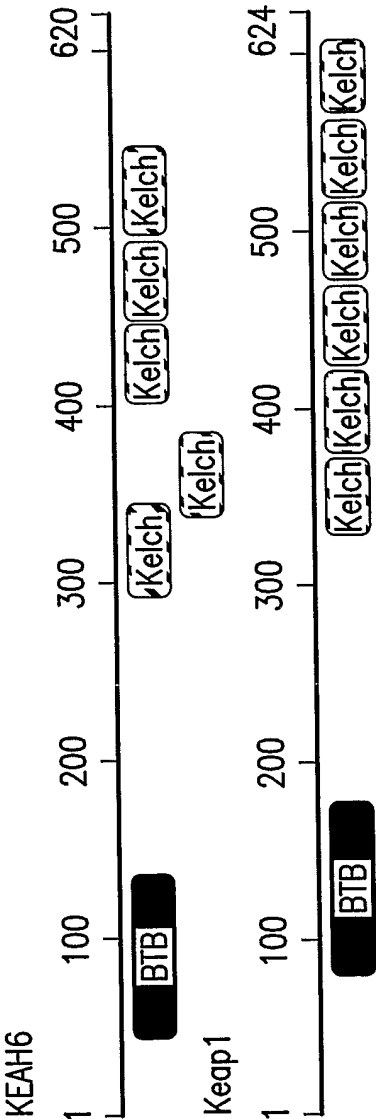


FIG.5



Score = 187 bits (475), Expect = 1e-45
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FIG.6

SEQUENCE LISTING

<110> Merck & Co., Inc.
 Majercak, John M.
 Ray, William J.
 Stone, David J.

<120> METHOD FOR IDENTIFYING MODULATORS OF
 KEAH6 USEFUL FOR TREATING ALZHEIMER'S DISEASE

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