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(71) Applicant *(for all designated States except US)*: **BLANCHETTE ROCKEFELLER NEUROSCIENCES INSTITUTE** [US/US]; One Medical Center Drive, Morgantown, WV 26506 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **KHAN, Tapan, K.** [IN/US]; 710 Timberline, Morgantown, WV 26505 (US). **ALKON, Daniel, L.** [US/US]; 2 Seven Locks Court, Bethesda, MD 20816 (US).

(74) Agent: **BOSCH, Michele, C.**; Finnegan, Henderson, Farabow, Garrett & Dunner, LLP, 901 New York Ave., N.W., Washington, D.C. 20001-4413 (US).

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(54) Title: ALZHEIMER'S DISEASE-SPECIFIC ALTERATIONS OF PROTEIN KINASE C EPSILON (PKC-EPSILON) PROTEIN LEVELS

(57) Abstract: The present invention relates to methods of diagnosing Alzheimer's Disease in a human patient by detecting alterations in the ratio of PKC epsilon protein levels in a human patient compared with PKC epsilon levels in a control subject. The Alzheimer's disease- specific molecular biomarkers disclosed herein are useful for the diagnosis of Alzheimer's disease and for screening methods for the identification of compounds for treating or preventing Alzheimer's disease. The present invention also provides methods for elevating PKC epsilon protein levels comprising the steps of contacting one or more human cells with an amount of a PKC activator effective to elevate PKC epsilon levels compared to an uncontacted human cell.

ALZHEIMER'S DISEASE-SPECIFIC ALTERATIONS OF PROTEIN KINASE C EPSILON (PKC-EPSILON) PROTEIN LEVELS

[0001] The present application claims priority to US Provisional Application Serial No. 61/306,922 filed February 22, 1010 and US Provisional Application Serial No.61/362,512 filed July 8, 2010 and US Provisional Application Serial No. 61/362,893 filed July 9, 2010, the disclosures of which are hereby incorporated herein by reference in their entireties.

FIELD OF THE INVENTION

[0002] The present invention relates to methods of diagnosing Alzheimer's Disease in a human patient by detecting alterations in the ratio of PKC epsilon protein levels in a human patient compared with PKC epsilon levels in a control subject. The Alzheimer's disease-specific molecular biomarkers disclosed herein are useful for the diagnosis of Alzheimer's disease and for screening methods for the identification of compounds for treating or preventing Alzheimer's disease. The present invention also provides methods for elevating PKC epsilon protein levels comprising the steps of contacting one or more human cells with an amount of a PKC activator effective to elevate PKC epsilon levels compared to an uncontacted human cell.

BACKGROUND OF THE INVENTION

[0003] Alzheimer's disease (AD), the most common form of dementia, begins with the loss of recent memory and is associated with two main pathological hallmarks in the brain: extracellular amyloid plaques and intracellular neurofibrillary

tangles. These are typically associated with a significant loss of synapses. Amyloid plaques are formed by the aggregation of Aβ peptide oligomers which are generated from cleavage of the amyloid precursor protein (APP) by the β- secretase and γ- secretase pathway, while α secretase generates the non-toxic, synaptogenic soluble APP-α. Accumulated observations indicate that Protein kinase C (PKC) isozymes -α and -ε directly activate the α- secretase mediated cleavage of APP directly (Slack et al., 1993; Kinouchi et al., 1995; Jolly-Tornetta and Wolf 2000; Yeon et al., 2001, Lanni et al., 2004), and/or indirectly through phosphorylation of the extracellular signal regulated kinase (ERK1/2) (Devari et al., 2006, Alkon et al., 2007). Many observations have also indicated that PKC signaling pathways regulate important events in neurodegenerative pathophysiology of AD such as the endothelin converting enzyme (ECE)-mediated degradation of Aβ (Nelson et al., 2009). *In vivo* over-expression of PKC-ε in AD-transgenic mice reduced amyloid plaques (Choi et al., 2006). Other studies have provided evidence that AD specific pathological abnormalities can be found in tissues other than brain which include blood, skin fibroblasts, and ocular tissues (Gurreiro et al., 2007, Ray et al., 2007). In AD skin fibroblasts, for example, defects were found of specific K^+ channels (Etcheberrigaray et al., 1993; 1994), PKC isozymes (Govoni et al., 1993, Favit et al., 1998), Ca^+ signaling (Ito et al., 1994), MAP kinase Erk1/2 phosphorylation (Zhao et al., 2002; Khan and Alkon, 2006), and PP2A (Zhao et al., 2003). For familial AD patients, skin fibroblasts showed enhanced secretion of Aβ (Citron et al., 1994; Johnston et al., 1994) while AD-specific reduction of specific K+ channels was induced by $A\beta_{1-40}$ in normal human fibroblasts (Etcheberrigaray, et al., 1993; 1994). Recently, an autopsy confirmed, internally controlled, phosphorylated

Erk1/2 peripheral biomarker in skin fibroblasts was shown to have promising sensitivity and specificity (Khan and Alkon, 2006; 2010). Still other studies have suggested deficits of PKC in particular brain regions of AD patients (Masliah et al., 1991). Finally, it has also been recently demonstrated that pharmacologic activators of PKC-α and -ε can protect two different strains of AD mice from all of the pathologic and cognitive abnormalities characteristics of AD (Hongpaisan et al., 2011). Consistent with these observations, PKC -α and –ε were found to be significantly reduced in AD transgenic mice and were restored to normal levels by treatment with pharmacologic activators of PKC-α and –ε (Hongpaisan et al., 2011).

[0004] Collectively, these and other previous studies have two important implications: I. AD has systemic pathologic expression with symptomatic consequences limited to brain function, and II. PKC isozymes particularly -α and -ε , play a critical role in regulating the major aspects of AD pathology including the loss of synapses, the generation of Aβ and amyloid plaques, and the GSK-3β- mediated hyperphosphorylation of tau in neurofibrilliary tangles. For these reasons we analyzed the PKC-ε in skin fibroblasts from AD, age-matched controls (AC) and non-AD dementia (non-ADD) patients at the steady state levels. This report reveals that PKC-ε as well as changes in these levels induced by application of soluble Aβ oligomers may provide a diagnostic basis for AD in peripheral tissues.

[0005] There exists a need for highly sensitive and highly specific tests to diagnose Alzheimer's disease and to screen for compounds useful in the treatment and prevention of Alzheimer's disease. The present inventors have identified, for the first time, unique Alzheimer's disease-specific molecular biomarkers useful for the diagnosis

of Alzheimer's disease in a highly sensitive and highly specific manner compared to previously known diagnostic tests. Thus, the unique Alzheimer's disease-specific molecular biomarkers disclosed herein serve as the basis for diagnostic methods having a high degree of sensitivity and specificity for the detection and diagnosis of Alzheimer's disease. The unique Alzheimer's disease-specific molecular biomarkers of the present invention are also useful in screening methods to identify compounds which may be used as therapeutic agents in the treatment and prevention of Alzheimer's disease. The inventors have also discovered methods for elevating PKC epsilon protein levels in human patients.

SUMMARY OF THE INVENTION

[0006] The present invention is based on the surprising finding that PKC epsilon levels are lower in Alzheimer's Disease subjects (AD) than in in age matched controls (AC). In certain embodiments, the invention is directed to a method of diagnosing Alzheimer's Disease in a human subject, said method comprising the steps of: a) determining the PKC epsilon level in said human subject; and b) comparing the PKC epsilon level in said human subject to the PKC epsilon level in a control subject; wherein said method is indicative of Alzheimer's Disease in said human subject if the PKC epsilon level in said human subject is lower than the PKC epsilon level in said control subject.

[0007] In certain embodiments of the diagnostice methods said PKC epsilon level are measured in one or more cells. In certain embodiments said PKC epsilon level is a PKC epsilon protein level or a PKC epsilon activity level. In certain

embodiments, the PKC epsilon level is measured by RT-PCR. In certain embodiments, the control subject does not have Alzheimer's Disease. In certain embodiments, the diagnostice methods of the present invention are conducted *in vitro*.

[0008] In certain preferred embodiments of the invention, said one or more cells is a fibroblast, buccal mucosal, neuron, or blood cell.

[0009] In certain embodiments, the measuring or determining steps of the level of PKC epsilon steps comprises a method selected from the group consisting of radioimmunoassay, Western blot assay, immunofluorescent assay, enzyme immunoassay, immuno-precipitation, chemiluminescent assay, immunohistochemical assay, dot blot assay and slot blot assay.

[0010] In certain embodiments, the absence of Alzheimer's Disease in said human subject is indicated if said PKC epsilon level in said human subject is greater than or equal to the PKC epsilon level in said control subject.

[0011] In certain preferred embodiments, the invention is directed to a method of diagnosing Alzheimer's Disease in a human subject comprising the steps of: a) obtaining one or more cells from a human subject; b) determining the PKC epsilon level in said one or more cells; c) contacting said one or more cells of step (a) with an agent that is a PKC epsilon activator; d) determining the PKC epsilon level in said one or more cells in step (c) after said contacting in step (c); wherein Alzheimer's Disease is indicated in said human subject if the PKC epsilon level determined in step (d) is greater that the PKC epsilon level determined in step (b).

[0012] In certain embodiments, the absence of Alzheimer's Disease in said human subject is indicated if said PKC epsilon level in determined in step (d) is equal to or less than the PKC epsilon level determined in step (b).

[0013] In certain embodiments, the invention is directed to a method of determining or monitoring Alzheimer's Disease progression in a human subject comprising the steps of: a) determining the PKC epsilon level in said human subject; b) comparing the PKC epsilon level in said human subject to the PKC epsilon level in a control subject; and c) determining or monitoring said Alzheimer's Disease progression based on said comparison in step (b).

[0014] In certain embodiments, the PKC epsilon level in said human subject decreases as Alzheimer's Disease progresses over time.

[0015] In certain embodiments, the PKC epsilon level increases in said human subject as Alzheimer's Disease progression is reversed.

[0016] In certain preferred embodiments, the invention is directed to methods for elevating the PKC epsilon protein level in a cell, comprising the step of contacting one or more human cells with an amount of a PKC activator effective to elevate the PKC epsilon protein level in said cell compared to an uncontacted human cell.

[0017] In certain embodiments, said human cell is a fibroblast, buccal mucosal, neuron, or blood cell. In certain embodiments said PKC activator is a macrocyclic lactone. In certain embodiments, said macrocyclic lactone is a bryostatin. In certain embodiments, said bryostatin is bryostatin-1. In certain embodiments, said PKC epsilon level is a PKC epsilon protein level or a PKC epsilon activity level.

[0018] In certain embodiments, the invention is directed to methods of diagnosing Alzheimer's Disease in a human subject comprising the steps of: a) obtaining one or more cells from a human subject; b) determining the PKC epsilon level in said one or more cells; c) contacting said one or more cells of step (a) with an Aβ peptide; d) determining the PKC epsilon level in said one or more cells in step (c) after said contacting in step (c); wherein Alzheimer's Disease is indicated in said human subject if the PKC epsilon level determined in step (d) is not significantly different from the PKC epsilon level determined in step (b).

[0019] In certain embodiments, the absence of Alzheimer's Disease in said human subject is indicated if said PKC epsilon level in determined in step (d) is less than the PKC epsilon level determined in step (b).

[0020] In certain embodiments, the invention is directed to kits comprising one or more antibodies specific for PKC epsilon. In certain embodiments said kit may comprise a PKC activator. In certain embodiments, said kit may comprise a PKC epsilon activator. In certain embodiments, said kit may comprise one or more oligonucleotides specific for a gene encoding PKC epsilon.

[0021] In certain embodiments, the invention is directedc to a kit comprising one or more oligonucleotides specific for a gene encoding PKC epsilon. In certain embodiments, said kit may comprise a PKC activator. In certain embodiments, said kit may comprise a PKC epsilon activator.

[0022] In certain embodiments, the invention is directed to a method of identifying a compound useful for the treatment of Alzheimer's Disease comprising: a) obtaining one or more cells from an Alzheimer's Disease subject; b) determining the

PKC epsilon level in said one or more cells; c) contacting said cells with a candidate compound; d) determining the PKC epsilon level in said one or more cells after said contacting step (c); wherein said candidate compound is identified as a compound useful for the treatment of Alzheimer's Disease if the PKC epsilon level determined in step (d) is greater than the PKC epsilon level determined in step (b).

[0023] Protein kinase C (PKC) isozymes particularly -α and -ε, play a critical role in regulating major aspects of AD pathology including the loss of synapses, the generation of Aβ and amyloid plaques, and the GSK-3β-mediated hyperphosphorylation of tau in neurofibrilliary tangles. Evidence of AD-specific signaling deficits has been previously found in peripheral tissues such as blood, skin fibroblasts, and ocular tissues. PKC-ε is an accurate AD Biomarker in AD skin fibroblasts.

[0024] In certain embodiments, basal protein levels of PKC-ε may be measured by western blot, immuno- fluorescence and at the transcript level by RT-PCR in cultured skin fibroblasts of AD patients, age-matched control (AC) cases, and non-AD dementia patients. Eleven AC, and ten AD subjects are selected both from sporadic and familial cases with the presence of amyloid plaques and neurofibrillary tangles in brain at autopsy (9 autopsy confirmed out of 10 AD cases). Eight inherited Huntington's disease (HD) patients with genetic evidence of non-AD characteristics, one Parkinson's disease patient, and one fronto-temporal dementia patient are included to establish that the deficiency of PKC-ε is due to only AD pathology.

[0025] PKC-ε levels in all the AD fibroblasts are found lower than the AC and non-AD dementia fibroblasts. The average PKC-ε in AD (0.501 ± 0.021, A.U.) is

found ~40% lower than the AC (0.857 ± 0.036, A.U.), and much lower than non-AD dementia (1.040 ± 0.288, A.U.) cases in western blots when normalized with respect to beta tubulin. A similar change is also found after immunofluorescence analysis. The mRNA level of PKC-ε (AC: 0.904 ± 0.103, AD: 0.530 ± 0.061) is also found to be lowered than that of AD patients. After oligomeric Aβ application to skin fibroblasts, the PKC-ε levels decreases in fibroblasts from AC, but not AD patients, indicating a pathophysiological Aβ effect on PKC-ε.

[0026] The inventors find, that PKC-ε levels are significantly lowered in the AD cultured skin fibroblasts compared to healthy AC and non-AD dementia cases. PKC-ε is a peripheral diagnostic biomarker and a therapeutic target for AD.

BRIEF DESCRIPTION OF THE FIGURES

[0027] **Figure 1A and 1B:** PKC-ε expression in cultured human fibroblasts from age-matched controls (AC), Alzheimer's disease (AD), and non-AD dementia. Values are means ± SEM of three independent experiments.

[0028] Figure 1A: Immunoreactivity of PKC-ε and β-tubulin in AC, AD, and non-AD dementia fibroblasts. AC1, AC2, and AC3 (AG07714, AG11734 and AG12927) are age matched control fibroblasts, AD1, AD2 and AD3 (AG06844, AG04159 and AG08245) are Alzheimer's disease fibroblasts and HD1 and HD2 (GM06274, GM04198) are Huntington's disease fibroblasts, respectively.

[0029] Figure 1B: Graphical representation of normalized densitometric ratios of PKC-ε to β-tubulin in eleven AC, ten AD, eight HD, one Parkinson's disease (PD) and one Frontotemporal dementia (FT). In AC cells the ratios varied between 0.7 -

1.2 (Y-axis), in the non-AD dementia the ratios varied between 0.72 - 1.3 (with two exceptions HD6 and HD8) while in AD the ratios of all the cell lines were below 0.6. Inset in Panel B: Mean values were 0.857 ± 0.0361 (SEM) in AC cells, 1.040 ± 0.288 in non-AD dementia and 0.501 ± 0.021 in AD cells. PKC-ε was significantly lower in AD compared to AC ($p<0.0001$) and non-AD dementia ($p= 0.0394$). The mean of AC11 (0.6213±0.040) was the lowest among the ACs. However, it was also significantly different ($P=0.0162$) when compared with all AD cases.

[0030] **Figure 2A and 2B:** Immuno fluorescence detection of PKC-ε in cultured human fibroblasts from age matched control (AC) and Alzheimer's disease (AD) fibroblasts.

[0031] Figure 2A: Confocal micrographs of age-matched control (AC), and Alzheimer's disease fibroblasts (AD). Green channel (FITC) represents PKC-ε, the blue channel is (DAPI) nuclear stain indicator, and the third channel represents a merged image. The Mean fluorescence intensity (MFI) from green (for PKC-ε) was measured from all cells for 5 different fields from each of five AC's and AD's fibroblasts. Values are mean ± SEM.

[0032] Figure 2B: Graphical representation of MFI (mean fluorescence intensity) of PKC-ε from 5 AC cases (AG07714, AG11734, AG05840, AG06242 and AG12927) and 5 AD cases (AG06844, AG04159, AG06840, AG05770 and AG08245. In AC cells the MFI varied between 15- 20A.U. (Y-axis), while in AD the range is in between 7-10. The average intensity of PKC-ε in AC's and AD were 18.092 ± 2.087 and 9.110 ± 1.420, respectively.

[0033] **Figure 3A and 3B:** RT-PCR analysis of PKC-ε.

[0034] Figure 3A: mRNA was isolated from three AC, three AD and two HD's cases. RT-PCR amplicons of PKC-ε, and β-tubulin were run on E-Gels and imaged on a Fuji gel scanner. (AC1, AC2 and AC3: AG11363, AG09977 and AG12998, respectively; AD1, AD2 and AD3: AG06263, AG10788 and AG08259, respectively; HD1 and HD2:GM02165 and GM04226, respectively).

[0035] Figure 3B: (a). Histogram representing the normalized value of PKC ε with respect to β-tubulin for three AC's, three AD's and two HD's. Values represent mean ± SEM of three independent experiments. (b) The mean PKC-ε mRNA level of the AD cells were significantly ($p < 0.0033$) lower than the AC cells (AC: 0.904 ± 0.103, AD: 0.530 ± 0.061 and HD: 0.701 ± 0.143).

[0036] **Figure 4A, 4B and 4C:** Soluble Aβ oligomers induce Alzheimer's PKC-ε phenotype of human fibroblasts.

[0037] Figure 4A: SDS – PAGE analysis of the synthesized Aβ oligomers from $A\beta_{1-42}$. Lane M: Protein molecular weight marker, Lane oAβ: Soluble Aβ oligomers.

[0038] Figure 4B: Soluble Aβ oligomer (500nM) treatment decreases the PKC-ε in all age-matched control skin fibroblasts (five AD and five AC cases). Mean normalized densitometric values of PKC-ε were calculated from five different cell lines (Aβ oligomers treated and untreated skin fibroblasts). In each case the AC value was calculated considering the AD mean value as one. ACs showed significant decrease in PKC-ε expression after Aβ treatment. (p values are 0.0044, 0.0035, 0.0005, 0.0330 and 0.0253 for AC1, AC2, AC3, AC4 and AC5, respectively), while AD cases did not show decrease in expression.

[0039] Figure 4C: Aβ oligomer treatment changed the AC to an AD phenotype. PKC-ε levels showed no significant difference in Aβ oligomer treated AC and AD cells, while in untreated cells AD showed a 40% reduced expression compared to AC (P=0.0292).

[0040] **Figure 5:** Interaction of PKC-ε with Aβ, implication in Alzheimer's disease. In AD pathology the over production Aβ by higher β-,γ- sectretase activity and lower α-secretase activity decrease the amount of PKC-ε. On the other hand PKC- α and PKC-ε increase the α-secretase activity, PKC-ε also increases the activity of Aβ degrading enzymes, particularly ECE (endothelin converting enzyme).

[0041] **Figure 6:** Structures of molecules contemplated for use according to the present invention (BR-101 through BR-118).

[0042] **Figure 7:** Schematic diagram of reduction of PKC epsilon level over time as a function of Alzheimer's Disease progression; or severity of cognitive impairment; or disease duration. PKC epsilon level may be an activity level, the protein level in one or more cells or transcript level measured, for example, by RT-PCR.

[0043] **Figure 8:** Bryostatin prevents the loss of PKCε in perforated fibers in Tg2576 mice (5x FAD)

[0044] **Figure 9:** PKCε in perforated fibers with and without bryostatin.

DETAILED DESCRIPTION OF THE INVENTION

[0045] As used herein, the term "PKC epsilon level" includes, but is not limited to, any one or more of the following: the enzymatic activity of PKC epsilon, the amount of PKC epsilon protein, or the amount of RNA encoding PKC epsilon.

[0046] A "fatty acid" is a carboxylic acid with an unbranched aliphatic chain containing from about 4 to 30 carbons; most long chain fatty acids contain between 10 and 24 carbons. Fatty acids can be saturated or unsaturated. Saturated fatty acids do not contain any double bonds or other functional groups along the chain. Unsaturated fatty acids contain one or more alkenyl functional groups, *i.e.*, double bonds, along the chain. The term "polyunsaturated fatty acid" or "PUFA" means a fatty acid containing more than one double bond. There are three classes of PUFAs, omega-3 PUFAs, omega-6 PUFAs, and omega-9 PUFAS. In omega-3 PUFAs, the first double bond is found 3 carbons away from the last carbon in the chain (the omega carbon). In omega-6 PUFAs the first double bond is found 6 carbons away from the chain and in omega-9 PUFAs the first double bond is 9 carbons from the omega carbon.

[0047] PUFAs are also called "polyenoic fatty acids." As used herein, the term PUFA includes both naturally-occurring and synthetic fatty acids. A major source for PUFAs is from marine fish and vegetable oils derived from oil seed crops, although the PUFAs found in commercially developed plant oils are typically limited to linoleic acid and linolenic acid (18:3 delta 9,12,15).

[0048] A "*cis*-PUFA" is one in which the adjacent carbon atoms are on the same side of the double bond.

[0049] The abbreviation X:Y indicates an acyl group containing X carbon atoms and Y double bonds. For example, linoleic acid would be abbreviated 18:2.

[0050] A "methylene-interrupted polyene" refers to a PUFA having two or more *cis* double bonds separated from each other by a single methylene group.

[0051] A "non-methylene-interrupted polyene," or "polymethylene-interrupted fatty acid," refers to a PUFA having two or more *cis* double bonds separated by more than one methylene group.

[0052] A "monounsaturated fatty acid" (MUFA) is a fatty acid that has a single double bond in the fatty acid chain and all the remaining carbon atoms in the chain are single-bonded. Exemplary MUFAs include oleic acid, myristoleic acid and palmitoleic acid.

[0053] A "*cis*-monounsaturated fatty acid" means that adjacent hydrogen atoms are on the same side of the double bond.

[0054] Conjugated fatty acids such as conjugated linoleic acid (9-*cis*,11-trans-octadecadienoic acid) possess a conjugated diene, that is, two double bonds on adjacent carbons. Some evidence suggests that conjugated linoleic acid has antitumor activity.

[0055] Exemplary PUFAs include lineoleic acid (9,12-octadecadienoic acid); γ-linolenic acid (GLA; 6,9,12-octadecatrienoic acid); α-linolenic acid (9,12,15-octadecatrienoic acid); arachidonic acid (5,8,11,14-eicosatetraenoic acid); eicosapentanoic acid (EPA; 5,8,11,14,17-eicosapentanoic acid); docosapentaenoic acid (DPA; 7,10,13,16,19-docosapentaenoic acid); docosahexaenoic acid (DHA; 4,7,10,13,16,19-docosahexanoic acid); and stearidonic acid (6,9,12,15-octadecatetraenoic acid).

[0056] As used herein, the term "cyclopropane" refers to a cycloalkane molecule with the molecular formula C3H6, consisting of three carbon atoms linked to each other to form a ring, with each carbon atom bearing two hydrogen atoms.

[0057] An "epoxide" refers to a cyclic ether with three ring atoms.

[0058] As used herein, a "PUFA derivative" refers to a PUFA, or alcohol or ester thereof, in which at least one of the double bonds has been cyclopropanated or epoxidized.

[0059] As used herein, a "MUFA derivative" refers to a MUFA, or alcohol or ester thereof, in which the double bond has been cyclopropanated or epoxidized.

[0060] "Selective activation" of PKCε means that the PUFA derivative compound of the present invention activates PKCε to a greater detectable extent than any other PKC isozyme. In specific embodiments, the PUFA derivative activates PKCε at least 1-fold, 2-fold or 5-fold over the other PKC isozymes as measured using *e.g.*, the PKC activation assay described herein. Upon activation, protein kinase C enzymes are translocated to the plasma membrane by RACK proteins (membrane-bound receptor for activated protein kinase C proteins). In general, upon activation, protein kinase C enzymes are translocated to the plasma membrane by RACK proteins. Other indications of PKC activation include phosphorylation at specific C-terminal serine/threonine residues by phosphatidylinositol-trisphosphate-dependent kinase (PDK1), with at least two additional phosphorylations and/or autophosphorylations of well-conserved sequences in each enzyme of the PKC family. Activation of PKC is described in Sun and Alkon, *Recent Patents CNS Drug Discov.* 2006;1(2):147-56.

[0061] "Neurodegeneration" refers to the progressive loss of structure or function of neurons, including death of neurons.

[0062] For purposes of the present invention, a "neurological disease" refers to any central nervous system (CNS) or peripheral nervous system (PNS) disease that is associated with the β-amyloidogenic processing of APP. This may result in neuronal or glial cell defects including but not limited to neuronal loss, neuronal degeneration, neuronal demyelination, gliosis (*i.e.*, astrogliosis), or neuronal or extraneuronal accumulation of aberrant proteins or toxins (*e.g.*, Aβ).

[0063] One exemplary neurological disease is Alzheimer's Disease (AD). Another exemplary neurological disease is congophilic angiopathy (CAA), also referred to as cerebral amyloid angiopathy.

[0064] The term "Alzheimer's Disease" or "AD" refers to any condition where Aβ deposition will eventually accumulate in the cells of the central nervous system. In one, non-limiting embodiment, Aβ, particularly Aβ1-42, peptide is formed from the β-amyloidogenic metabolism of APP. AD may be heritable in a Familial manifestation, or may be sporadic. Herein, AD includes Familial, Sporadic, as well as intermediates and subgroups thereof based on phenotypic manifestations.

[0065] Another neurological disease is Down syndrome (DS). Subjects with DS invariably develop (in their third or fourth decade) cerebral amyloid (Aβ) plaques and neurofibrillary tangles (NFTs), the characteristic lesions of AD. Recent studies have shown that the Aβ42 is the earliest form of this protein deposited in Down syndrome brains, and may be seen in subjects as young as 12 years of age, and that soluble Aβ can be detected in the brains of DS subjects as early as 21 gestational weeks of age, well preceding the formation of Aβ plaques. Gyure et al., *Archives of Pathology and Laboratory Medicine*. 2000; 125:. 489–492.

[0066] As used herein, the term "subject" includes a mammal.

[0067] The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce untoward reactions when administered to a subject. Preferably, as used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "pharmaceutically acceptable carrier" means a chemical composition with which the active ingredient may be combined and which, following the combination, can be used to administer the active ingredient to a subject and can refer to a diluent, adjuvant, excipient, or vehicle with which the compound is administered.

[0068] The terms "therapeutically effective dose" and "effective amount" refer to an amount of a therapeutic agent that results in a measurable therapeutic response. A therapeutic response may be any response that a user (*e.g.*, a clinician) will recognize as an effective response to the therapy, including improvement of symptoms and surrogate clinical markers. Thus, a therapeutic response will generally be an amelioration or inhibition of one or more symptoms of a disease or condition *e.g.*, AD. A measurable therapeutic response also includes a finding that a symptom or disease is prevented or has a delayed onset, or is otherwise attenuated by the therapeutic agent.

[0069] The terms "about" and "approximately" shall generally mean an acceptable degree of error for the quantity measured given the nature or precision of the measurements. Typical, exemplary degrees of error are within 20 percent (%), preferably within 10%, and more preferably within 5% of a given value or range of values.

Alternatively, and particularly in biological systems, the terms "about" and "approximately" may mean values that are within an order of magnitude, preferably within 5-fold and more preferably within 2-fold of a given value. Numerical quantities given herein are approximate unless stated otherwise, meaning that the term "about" or "approximately" can be inferred when not expressly stated.

PKC-ε levels are lower in AD fibroblasts

[0070] In this study population we have included six sporadic (late onset, without family history,) and four familial (early onset) cases of AD to test that PKC-ε is dysfunctional in both sporadic and familial cases and is a hallmark of AD pathological signaling. Immunoblot analysis of 10 AD cell lines, 11 age AC cell lines and 10 non-AD dementia fibroblasts revealed that the PKC-ε levels in the AD samples were lower by approximately 40% compared to the AC (Fig. 1). The average normalized ratio of PKC-ε against β-tubulin was 0.857 ± 0.036 (SEM) in AC cases (n=11), 1.040 ± 0.288 in non-AD dementia (n=10), and 0.501 ± 0.021 in AD cells (n=10). The PKC-ε levels were significantly lower in the AD fibroblasts ($p<0.0001$) compare to AC cases. The mean basal PKC-ε level for the AC11 case (0.6213±0.040) was the lowest among all ACs (Figure 1B). However, the basal level of PKC-ε of AC11 was also statistically significant when compared separately with all AD cases (P=0.0162).

[0071] Data from western blot analysis were supported by immunofluorescence analysis of stained fibroblasts that demonstrated distinct differences in intensity of PKC-ε tagged with FITC between AC and AD cells (Fig. 2). The average intensity of PKC-ε in AC and AD cells were 18.092 ± 2.087 and 9.110 ± 1.420,

respectively. To test for dysfunctional PKC-ε at the transcript level, RT-PCR experiments were conducted. The average mRNA levels of three AD, three AC and two HD cell lines were measured (Fig. 3). All PKC-ε mRNA levels were normalized with β-tubulin mRNA levels of corresponding cell lines. The normalized average mRNA level of AD cells were significantly ($p < 0.003$) lower than the AC and HD cells (AC: 0.904 ± 0.103, AD: 0.530 ± 0.061 and HD: 0.701 ± 0.143) (Fig. 3).

Treatment of skin fibroblasts with oligomeric Aβ

[0072] Oligomeric Aβ synthesized by the method described earlier produced high molecular weight oligomers with molecular weight of > 100kDa size (Fig. 4A). These oligomers were reported to be highly toxic and had similarities to those found in the AD brain (Nouguchi et al., 2009). To establish the pathophysiological relevance of AD, exogenous toxic oligomeric $A\beta_{1-42}$ was added to the normal fibroblasts (AC) and the impact on PKC-ε expression level was assessed at basal state. After treatment with oligomeric Aβ, the PKC-ε levels were found decreased in the AC cases, while the AD cases showed no statistical difference. Average of the mean of three independent experiments from five different AC and AD patients was calculated following oligomeric Aβ treatment and was compared to the untreated cells. Untreated AC and AD cells showed a difference of ~40% among them, while treated AC and AD cells demonstrated no difference in PKC-ε expression or were sometimes higher for the AD cases after Aβ treatment .

[0073] The present invention relates, in certain aspects, to methods of diagnosing Alzheimer's disease in human cells taken from subjects that have been identified for testing and diagnosis. The diagnosis is based upon the discovery of unique

Alzheimer's disease-specific molecular biomarkers. In certain aspects, the invention is directed to methods of monitoring Alzheimer's disease progression and to screening methods for the identification of lead compounds for treating or preventing Alzheimer's disease.

[0074] Because direct access to neurons in the brains of living human beings is impossible, early diagnosis of Alzheimer's disease is extremely difficult. By measuring the Alzheimer's disease-specific molecular biomarkers disclosed herein, the present invention provides highly practical, highly specific and highly selective tests for early diagnosis of Alzheimer's disease. In addition, the Alzheimer's disease-specific molecular biomarkers described herein provide a basis for following disease progression and for identifying therapeutic agents for drug development targeted to the treatment and prevention of Alzheimer's disease.

[0075] The inventors have found a unique molecular biomarker for Alzheimer's disease using peripheral (non-CNS) tissue that is useful in diagnostic assays that are highly sensitive and highly specific for the diagnosis of Alzheimer's disease. A great advantage of the instant invention is that the tissue used in the assays and methods disclosed herein may be obtained from subjects using minimally invasive procedures, i.e., without the use of a spinal tap. Thus, one aspect of the invention is directed to an assay or test for the early detection of Alzheimer's disease.

[0076] In one embodiment, the invention is directed to methods for screening a test compound (or a lead compound) useful for the treatment or prevention of Alzheimer's disease wherein the methods comprise an in vitro assay.

[0077] In further embodiments of the invention, the protein kinase C activator is selected from the group consisting of bradykinin, bryostatin, bombesin, cholecystokinin, thrombin, prostaglandin F2 alpha and vasopressin. In further embodiments of the invention, the cells are peripheral cells. In still further embodiments of the invention, the peripheral cells are selected from the group consisting of skin cells, skin fibroblast cells, blood cells and buccal mucosa cells. In still further embodiments of the invention, the cells are not isolated from cerebral spinal fluid. In still further embodiments of the invention, the cells do not comprise cerebral spinal fluid. In still further embodiments of the invention, the cells are not obtained by a spinal tap or lumbar puncture. In still further embodiments of the invention, the protein kinase C activator is contacted with said cells in media comprising serum. In still further embodiments of the invention, the protein kinase C activator is contacted with said cells in serum-free media. In still further embodiments of the invention, the PKC epsilon proteins are detected by immunoassay. In still further embodiments of the invention, the immunoassay is a radioimmunoassay, a Western blot assay, an immunofluoresence assay, an enzyme immunoassay, an immunoprecipitation assay, a chemiluminescence assay, an immunohistochemical assay, an immunoelectrophoresis assay, a dot blot assay, or a slot blot assay. In still further embodiments of the invention, the measuring is done using a protein array, a peptide array, or a protein micro array.

[0078] In further embodiments of the invention, the PKC activator, or pharmaceutical composition, comprises any of the following compounds selected from the group consisting of DCP-LA; DCPLA methyl ester, DHA-CP6 methyl ester (BR-111); EPA-CP5 methyl ester (BR-114); AA-CP4 methyl ester (BR-115); DHA-CP6;

EPA-CP5; AA-CP4; Linolenyl alcohol cyclpropanated (BR-104); Linoleic alcohol cyclopropanated (BR-105); Elaidic alcohol cyclopropanated (BR-106); Elaidic acid cyclopropanated (BR-107); Oleyl alcohol cyclopropanated(BR-108); Vernolic acid cyclopropanated methyl ester (BR-109); Linolenic acid cyclopropanated (BR-118); Elaidic acid cyclopropanated methyl ester; Vernolic acid cyclopropanated; Linolenic acid cyclopropanated methyl ester;

[0079] 8-(2-((2-pentylcyclopropyl)methyl)cyclopropyl)octanoicacid (DCP-LA);

[0080] methyl 3-(2-((2-((2-((2-((2-((2-ethylcyclopropyl)methyl)cyclopropyl)methyl)cyclopropyl)methyl)-cyclopropyl)methyl)cyclopropyl)methyl)cyclopropyl)propanoate

[0081] methyl 3-(2-((2-((2-((2-((2-((2-ethylcyclopropyl)methyl)cyclopropyl)methyl)cyclopropyl)methyl)-cyclopropyl)methyl)cyclopropyl)methyl)cyclopropyl)propanoate (DHA-CP6 methyl ester);

[0082] methyl 4-(2-((2-((2-((2-((2-ethylcyclopropyl)methyl)cyclopropyl)methyl)cyclopropyl)-methyl)cyclopropyl)methyl)cyclopropyl)butanoate

[0083] methyl 4-(2-((2-((2-((2-((2-ethylcyclopropyl)methyl)cyclopropyl)methyl)cyclopropyl)-methyl)cyclopropyl)methyl)cyclopropyl)butanoate (EPA-CP5 methyl ester)

[0084] methyl 4-(2-((2-((2-((2-pentylcyclopropyl)methyl)cyclopropyl)methyl)-cyclopropyl)methyl)cyclopropyl)butanoate

[0085] methyl 4-(2-((2-((2-((2-pentylcyclopropyl)methyl)cyclopropyl)methyl)-cyclopropyl)methyl)cyclopropyl)butanoate (AA-CP4 methyl ester)

[0086] In the methods of the invention, the cells that are taken from the individual or patient can be any viable cells. Preferably, they are skin fibroblasts, but any other peripheral tissue cell (i.e. outside of the central nervous system) may be used in the tests of this invention if such cells are more convenient to obtain or process. Other suitable cells include, but are not limited to, blood cells such as erythrocytes and lymphocytes, buccal mucosal cells, nerve cells such as olfactory neurons, cerebrospinal fluid, urine and any other peripheral cell type. In addition, the cells used for purposes of comparison do not necessarily have to be from healthy donors.

[0087] The cells may be fresh or may be cultured (see, U.S. Patent No. 6,107,050, which is herein incorporated by reference in its entirety). In a specific embodiment, a punch skin biopsy can be used to obtain skin fibroblasts from a subject. These fibroblasts are analyzed directly using the techniques described herein or introduced into cell culture conditions. The resulting cultured fibroblasts are then analyzed as described in the examples and throughout the specification. Other steps may be required to prepare other types of cells which might be used for analysis such as buccal mucosal cells, nerve cells such as olfactory cells, blood cells such as erythrocytes and lymphocytes, etc. For example, blood cells can be easily obtained by drawing blood

from peripheral veins. Cells can then be separated by standard procedures (e.g. using a cell sorter, centrifugation, etc.) and later analyzed.

[0088] Thus, the present invention relates, in certain aspects, to methods for the diagnosis and treatment of Alzheimer's disease in a subject. The invention is also directed, in certain embodiments, to kits containing reagents useful for the detection or diagnosis of Alzheimer's disease. In certain aspects, the invention is directed to methods for screening to identify lead compounds useful for treating Alzheimer's disease as well as to methods of using these compounds or chemical derivatives of the lead compounds in pharmaceutical formulations to treat or prevent Alzheimer's disease in subjects in need thereof.

[0089] Protein kinase C activators that are specifically contemplated for use in the diagnostic methods, kits and methods of screening to identify compounds of the instant invention include, but are not limited to: Bradykinin; ε-APP modulator; Bryostatin 1; Bryostatin 2; DHI; 1,2-Dioctanoyl-sn-glycerol; FTT; Gnidimacrin, Stellera chamaejasme L.; (-)-Indolactam V; Lipoxin A4; Lyngbyatoxin A, Micromonospora sp.; Oleic acid; 1-Oleoyl-2-acetyl-sn-glycerol; 4α-Phorbol; Phorbol-12,13-dibutyrate; Phorbol-12,13-didecanoate; 4α-Phorbol-12,13-didecanoate; Phorbol-12-myristate-13-acetate; L-α-Phosphatidylinositol-3,4-bisphosphate, Dipalmitoyl-, Pentaammonium Salt; L-α -Phosphatidylinositol-4,5-bisphosphate, Dipalmitoyl-, Pentaammonium Salt; L-α -Phosphatidylinositol-3,4,5-trisphosphate, Dipalmitoyl-, Heptaammonium Salt; 1-Stearoyl-2-arachidonoyl-sn-glycerol; Thymeleatoxin, Thymelea hirsuta L.; insulin, phorbol esters, lysophosphatidylcholine, lipopolysaccharide, anthracycline dannorubicin and vanadyl sulfate. Also included are compounds known as "bryologues." Bryologues

are described, for example, in Wender et al. Organic letters (United States) May 12, 2005 ,7 (10) p1995-8; Wender et al. Organic letters (United States) Mar 17 2005 , 7 (6) p1177-80; Wender et al. Journal of Medicinal Chemistry (United States) Dec 16 2004, 47 (26) p6638-44. A protein kinase C activator may be used alone or in combination with any other protein kinase C activator in the diagnostic methods, kits and methods of screening compounds disclosed herein.

[0090] Bradykinin is a potent vasoactive nonapeptide that is generated in the course of various inflammatory conditions. Bradykinin binds to and activates specific cell membrane bradykinin receptor(s), thereby triggering a cascade of intracellular events leading to the phosphorylation of proteins known as "mitogen activated protein kinase" (MAPK). Phosphorylation of protein, the addition of a phosphate group to a Ser, Thr, or Tyr residue, is mediated by a large number of enzymes known collectively as protein kinases. Phosphorylation normally modifies the function of, and usually activates, a protein. Homeostasis requires that phosphorylation be a transient process, which is reversed by phosphatase enzymes that dephosphorylate the substrate. Any aberration in phosphorylation or dephosphorylation may disrupt biochemical pathways and cellular functions. Such

[0091] Immunoassays of the present invention for the detection of proteins may be immunofluorescent assays, radioimmunoassays, Western blot assays, enzyme immunoassay, immuno-precipitation, chemiluminescent assay, immunohistochemical assay, dot or slot blot assay and the like. (In "Principles and Practice of Immunoassay" (1991) Christopher P. Price and David J. Neoman (eds), Stockton Press, New York, New York, Ausubel et al. (eds) (1987) in "Current Protocols

in Molecular Biology" John Wiley and Sons, New York, New York). Detection may be by colorometric or radioactive methods or any other conventional methods known to those having skill in the art. Standard techniques known in the art for ELISA are described in Methods in Immunodiagnosis, 2nd Edition, Rose and Bigazzi, eds., John Wiley and Sons, New York 1980 and Campbell et al., Methods of Immunology, W.A. Benjamin, Inc., 1964, both of which are incorporated herein by reference. Such assays may be direct, indirect, competitive, or noncompetitive immunoassays as described in the art (In "Principles and Practice of Immunoassay" (1991) Christopher P. Price and David J. Neoman (eds), Stockton Pres, NY, NY; Oellirich, M. 1984. J. Clin. Chem. Clin. Biochem. 22: 895-904 Ausubel, et al. (eds) 1987 in Current Protocols in Molecular Biology, John Wiley and Sons, New York, New York.

[0092] As stated previously, the cells taken from the patient being diagnosed may be any cell. Examples of cells that may be used include, but are not limited to, skin cells, skin fibroblasts, buccal mucosal cells, blood cells, such as erythrocytes, lymphocytes and lymphoblastoid cells, and nerve cells and any other cell expressing PKC epsilon protein. Necropsy samples and pathology samples may also be used. Tissues comprising these cells may also be used, including brain tissue or brain cells. The cells may be fresh, cultured or frozen. Protein samples isolated from the cells or tissues may be used immediately in the diagnostic assay or methods for screening compounds or frozen for later use. In a preferred embodiment fibroblast cells are used. Fibroblast cells may be obtained by a skin punch biopsy.

[0093] Proteins may be isolated from the cells by conventional methods known to one of skill in the art. In a preferred method, cells isolated from a patient are

washed and pelleted in phosphate buffered saline (PBS). Pellets are then washed with "homogenization buffer" comprising 50 nM NaF, 1mM EDTA, 1 mM EGTA, 20 μg/ml leupeptin, 50 μg/ml pepstatin, 10 mM TRIS-HCl, pH = 7.4, and pelleted by centrifugation. The supernatant is discarded, and "homogenization buffer" is added to the pellet followed by sonication of the pellet. The protein extract may be used fresh or stored at -80°C for later analysis.

[0094] In the methods of the invention, the antibodies used in the disclosed immunoassays may be monoclonal or polyclonal in origin. The whole PKC epsilon protein or portions thereof used to generate the antibodies may be from natural or recombinant sources or generated by chemical synthesis. Natural Erk1/2 proteins can be isolated from biological samples by conventional methods. Examples of biological samples that may be used to isolate the PKC epsilon protein include, but are not limited to, skin cells, such as, fibroblasts, fibroblast cell lines, such as Alzheimer's disease fibroblast cell lines and control fibroblast cell lines which are commercially available through Coriell Cell Repositories, (Camden, N.J.) and listed in the National Institute of Aging 1991 Catalog of Cell Lines, National Institute of General Medical Sciences 1992/1993 Catalog of Cell Lines [(NIH Publication 92-2011 (1992)].

[0095] The present invention is also directed to methods to screen and identify substances useful for the treatment or prevention of Alzheimer's disease. According to this embodiment, substances which reverse or improve the Alzheimer's disease-specific molecular biomarkers described herein (i.e. back to levels found in normal cells) would be identified and selected as substances which are potentially useful for the treatment or prevention of Alzheimer's disease.

[0096] By way of example, one such method of screening to identify therapeutic substances would involve the steps of contacting sample cells from an Alzheimer's disease patient with a substance being screened in the presence of any of the protein kinase C activators disclosed herein and then measuring any of the Alzheimer's disease-specific molecular biomarkers disclosed herein. An agent that reverses or improves the Alzheimer's disease-specific molecular biomarker back to levels found in normal cells (i.e. cells taken from a subject without Alzheimer's disease) would be identified and selected as a substance potentially useful for the treatment or prevention of Alzheimer's disease.

[0097] The present invention is also directed to compositions useful for the treatment or prevention of Alzheimer's disease. Compounds identified using the screening methods described herein may be formulated as pharmaceutical compositions for administration to subjects in need thereof.

[0098] A pharmaceutical composition of the present invention or a compound (or a chemical derivative of a lead compound) identified using the screening methods disclosed herein can be administered safely by admixing with, for example, a pharmacologically acceptable carrier according to known methods to give a pharmaceutical composition, such as tablets (inclusive of sugar-coated tablets and film-coated tablets), powders, granules, capsules, (inclusive of soft capsules), liquids, injections, suppositories, sustained release agents and the like, for oral, subcutaneous, transdermal, transcutaneous or parenteral (e.g., topical, rectal or intravenous) administration.

[0099] Examples of pharmacologically acceptable carriers for use in the pharmaceutical compositions of the invention include, but are not limited to various conventional organic or inorganic carriers, including excipients, lubricants, binders and disintegrators for solid preparations, and solvents, solubilizers, suspending agents, isotonic agents, buffers, soothing agents, and the like for liquid preparations. Where necessary, conventional additives such as antiseptics, antioxidants, coloring agents, sweeteners, absorbents, moistening agents and the like can be used appropriately in suitable amounts.

[00100] A growing body of evidences suggests that a PKC signaling deficit is one of the major elements in causing the pathology of AD (Alkon et al., 2007, Liron et al., 2007, Choi et al., 2006). Previous findings have demonstrated that the distribution of PKC isozymes changes in the brains of AD patients (Shimohama et al., 1993, Masliah et al., 1990). PKC-α, PKC-γ and PKC-β were found lower in AD brains. Matsushima et al., 1996 have reported that in AD brain the PKC-ε level in both cytosolic and membranous fractions was found reduced, although PKC-δ and PKC-ξ levels were not changed, suggesting that among Ca2+-independent PKC isozymes, the alteration of PKC-ε is a specific event in AD brain and has a crucial role in AD pathophysiology. The major means of activating α-secretase mediated cleavage of APP is accomplished by PKC isozyme -α and -ε or indirectly through PKC mediated ERK1/2 or both (Alkon et al., 2006; Skovronsky et al., 2000; Diaz-Rodrigez et al., 2002; Robinson and Cobb, 1997). The greatest risk factor for sporadic AD is age and it is associated with differential distribution of PKC isozymes in brain, impaired translocation, and reduced

level of PKC anchoring protein RACK1, (receptor of activated protein kinase C) (Battani et al., 1997).

[00101] **PKC-ε signaling deficit related to AD:** According to the amyloid hypothesis, AD is caused by the aggregation and accumulation of Aβ peptide forming amyloid plaque generated by the β- and γ-secretase pathway. Studies with human skin fibroblasts have documented anomalies in PKC isozyme function between AD patients and age matched controls (Van Huynh et al., 1989, Etcheberrugaray et al., 1993, Govoni et al., 1993, Favit et al., 1998). However, there has been previously no evidence showing decreased PKC-ε levels in peripheral tissues such as blood cells or skin fibroblasts of AD patients. To investigate, whether the lowered basal level of PKC-ε is AD-specific, AD patients were selected from both sporadic and familial AD cases with the presence of amyloid plaques and neurofibrilliary tangles in brain at autopsy and also compared with two different sets of non-AD dementia controls such as: (i) eight inherited Huntington's disease (HD) patients with strong evidence of non-AD characteristics with genetic identification of HD, and (ii) one Parkinson's disease and one fronto-temporal dementia patient fibroblasts. Therefore, the PKC-ε deficits were not associated with other non-AD dementia pathology. The basal levels of PKC-ε may also decrease with age. However, this study clearly demonstrated that the eleven AC (age–matched controls) had significantly higher PKC-ε levels. Therefore, the lowered basal level of PKC-ε in AD skin fibroblasts is due to the Alzheimer's pathology and not aging itself.

[00102] AD is a disease involving multiple pathological deficits and PKC is one of the major mechanistic controllers of cell survival, differentiation, and regulation. Among PKC's, PKC-ε controls synaptogenesis. PKC-ε is also reported to

induce the transcription of low density receptor during cholesterol depletion (Mehta et al., 2002) and the LDL receptors have been suggested to play a role in transport and clearance of Aβ. In other studies, PKC ε activators were shown to enhance learning and memory as well as structurally specific synaptic changes in rat spatial maze learning (Hongpaisan and Alkon, 2007). Therefore, depletion in the total amount of PKC-ε in patients could lead to memory loss in AD. Furthermore, the transcript levels of the PKC-ε mRNA were also lower in the AD patient samples.

[00103] **Pathophysiological relevance of PKC-ε in AD**: It has been previously shown that PKC-α is degraded by Aβ treatment (Favit et al., 1998), and that Aβ alters the membrane translocation of PKC-α and PKC-ε in B103 cells upon phorbol ester treatment (Lanni et al., 2004). It has also been shown that overexpression of PKC-ε reduced Aβ levels in transgenic mice (Choi et al., 2006; Hongpaisan et al., 2011). Aβ oligomers which were > 100kDa molecular mass were found by to be highly toxic on primary rat neurons (Noguchi et al., 2009; Hoshi et al., 2003). Antibodies against these synthetic oligomers recognizes naïve amylospheroids from AD patient brain (Noguchi et al., 2009), and hence these oligomers are pathologically significant with the disease. We have demonstrated (Fig. 4B) that these highly toxic oligomers (>100kDa) affected the PKC-ε levels in AC fibroblasts and converted it to AD phenotype. Treatment of the AC cells with these oligomers reduced the expression level of PKC-ε while it did not affect the AD cells. The AC-AD ratio of normalized PKC-ε in treated cells was found ~1, while in untreated cells it was 1.4 (Fig. 4C).

[00104] In AD pathology, over production of Aβ by higher β-,γ- sectretase activity and lower α-secretase activity might result in decreased amount of PKC-ε while

on the other hand PKC- α and PKC-ε increase α-secretase activity, as well as PKC-ε increases the activity of Aβ degrading enzymes. Since PKC-ε levels are found significantly lower in the AD fibroblasts compared to AC's and non- AD dementia's, therefore, AD related dysfunction of PKC-ε signaling and decreased basal amounts of PKC-ε in skin fibroblasts supports the possibility of peripheral PKC-ε as a biomarker for AD and PKC-ε activators as therapeutic candidates. It is possible that the different forms of toxic Aβ oligomers affect the PKC-ε levels in the cells, which is responsible for regulating the endothelin converting enzyme (ECE), that degrades Aβ. These proteins play an important role in Aβ clearance. Thus, a reasonable hypothesis is that abnormal accumulation of Aβ due to higher β-,γ- secretase activity causes a decrease in PKC-ε that then participates in a feedback loop (Fig. 5) to cause further Aβ elevation and synaptic loss.

[00105] Deficiency of PKC-ε in AD fibroblasts and peripheral biomarker: Though the gold standard for diagnosis of AD is postmortem analysis of neuropathological parameters, various laboratories are trying to find an effective diagnosis using peripheral tissue with the advantage of non-invasiveness, easy availability, low cost and most importantly early detection of the disease. The findings disclosed herein show that PKC signaling deficit is behind most of the AD elements. In aged animals, the PKC function is compromised with age specific distribution of PKC isozymes in brain, reduced translocation and reduced level of the RACK1 protein (Battani et al., 1997) and age is the most important risk factor in the case of sporadic AD. Over expression of PKC-ε has been shown to reduce the level of Aβ in AD transgenic

mice (Choi et al., 2006). The inventors have surprisingly shown that PKC-ε is deficient in the peripheral cells of AD patients.

Table: 1. Patient population: Description and identification of the human dermal fibroblasts

	ID	Age	Sex	Description
1	AG06844	59 YR	Male	Autopsy confirmed familial type 3 AD; 11yrs of disease
2	AG04159	52 YR	Female	Autopsy confirmed familial type 3 AD; 40 yrs of disease
3	AG06840	56 YR	Male	Autopsy confirmed familial type 3 AD; 1 yr of disease duration.
4	AG08245	75 YR	Male	Autopsy confirmed AD with no family history; 7yrs of disease
5	AG05770	70 YR	Male	Autopsy confirmed AD with no family history; 7 &1/2 yrs disease
6	AG08527	61 YR	Male	Autopsy confirmed AD; 1 yr of disease
7	AG06263	67 YR	Female	Autopsy confirmed AD with no family history; 7yrs of disease
8	AG10788	87 YR		Autopsy confirmed AD, familial; 17yrs of disease. Homozygous for Apoe4
9	AG08259	90 YR	Male	Autopsy confirmed AD with no Family History, 3yrs of disease.
10	AG05810	79 YR	Female	The donor is clinically affected with severe late stage dementia, typical of AD. The APOE genotype of the donor subject is E3/E4
11	AG07714	56 YR	Female	Age matched control fibroblast
12	AG11734	50 YR	Female	Age matched control fibroblast
13	AG05840	55 YR	Female	Age matched control fibroblast
14	AG12927	66 YR	Female	Age matched control fibroblast
15	AG06242	71 YR	Male	Age matched control fibroblast
16	AG04461	66 YR	Male	Age matched control fibroblast
17	AG11363	74 YR	Female	Age matched control fibroblast
18	AG09977	63 YR	Female	Age matched control fibroblast
19	AG12998	65 YR	Male	Age matched control fibroblast
20	AG04560	59 YR	Male	Age matched control fibroblast
21	AG13358	72 YR	Female	Age matched control fibroblast
22	ND27760	55 YR	Female	Familial type 1 Parkinson's disease; Park1.
23	GM20926	35 YR	Female	Inclusion body Myopathy with early-onset Paget disease and Frontotemporal dementia
24	GM06274	56 YR	Female	Huntington's disease
25	GM02173	52 YR	Female	Huntington's disease
26	GM00305	56 YR	Female	Huntington's disease; 10 yrs of disease duration.
27	GM04198	63 YR	Female	Huntington's disease inherited.
28	GM05031	60 YR	Male	Huntington's disease inherited.
29	GM02165	57 YR	Male	Huntington's disease inherited. 11 yrs of disease
30	GM04226	74YR	Male	Huntington's disease inherited.
31	GM05030	56YR	Male	Huntington's disease inherited.

Activators of PKC Epsilon

[00106] PKCε is the isozyme that most effectively suppresses Aβ production. Racci et al., *Mol. Psychiatry.* 2003; 8:209-216; and Zhu et al., *Biochem. Biophys. Res. Commun.* 2001; 285: 997-1006. Thus, isoform specific PKC activators are highly desirable as potential anti-Alzheimer's drugs. Specific activators are preferable to compounds such as bryostatin that show less specificity because non-specific activation of PKCδ or β could produce undesirable side effects.

[00107] Moreover, PKCε is also expressed at very low levels in all normal tissues except for brain. Mischak et al., *J. Biol. Chem.* 1993; 268: 6090-6096; Van Kolen et al., *J. Neurochem.* 2008;104:1-13. The high abundance of PKCε in presynaptic nerve fibers suggest a role in neurite outgrowth or neurotransmitter release. Shirai et al., *FEBS J.* 2008; 275: 3988-3994). Therefore, effects of specific PKCε activators would be largely restricted to brain, and unlikely to produce unwanted peripheral side effects.

PUFAs as PKC Activators

[00108] Some PUFAs, such as arachidonic acid (see Fig. 6), have been known for many years to be natural activators of PKC. Docosahexaenoic acid (DHA) is also a known activator of PKC and has recently been shown to slow the accumulation of Aβ and tau proteins associated with the brain-clogging plaques and tangles implicated in AD. Sahlin et al., *Eur J Neurosci.* 2007; 26(4):882-9.

[00109] Kanno et al. described effect of 8-[2-(2-pentyl-cyclopropylmethyl)-cyclopropyl]-octanoic acid (DCP-LA), a newly synthesized linoleic acid derivative with cyclopropane rings instead of *cis*-double bonds, on protein kinase C

(PKC) activity. *Journal of Lipid Research.* 2007; 47: 1146-1156. DCP-LA activated PKCε, with a greater than 7-fold potency over other PKC isozymes. This indicates that DCP-LA is highly specific for PKCε. This compound also facilitated hippocampal synaptic transmission by enhancing activity of presynaptic acetylcholine receptors on the glutamatergic terminals or neurons. However, DCP-LA requires relatively high concentrations to produce its maximal effect.

[00110] WO 2002/50113 to Nishizaki et al., discloses carboxylic acid compounds and their corresponding salts having cyclopropane rings for LTP-like potentiation of synaptic transmission or for use as a cognition-enhancing drug or a drug to treat dementia. Their synthetic examples disclose preparation of esters but their experimental results teach the use of free acids. The reason is that the carboxylic acid group of the fatty acid starting material would react with the diethylzinc used in the Simmons-Smith reaction. The methyl ester acts as a protecting group and may be cleaved off by hydrolysis or allowed to remain as needed.

[00111] The caveats with the prior art finding include the necessity of administering high concentrations of to achieve the foregoing effects, non-specific activation of PKC isoforms, or rapid metabolism and sequestration of unmodified PUFAs into fat tissues and other organs where they are incorporated into triglycerides and chylomicrons. *J Pharmacobiodyn.* 1988;11(4):251-61. In addition use of unmodified PUFAs would have a myriad of adverse side effects. For example, arachidonic acid is a biochemical precursor to prostaglandins, thromboxanes, and leukotrienes, which have potent pro-inflammatory effects. This would be undesirable for treatment of Alzheimer's disease where the pathology likely involves inflammation. Other essential fatty acids

may also possess a multitude of other biological effects, including enhancement of nitric oxide signaling, anti-inflammatory effects, and inhibition of HMG-CoA reductase, which would interfere with cholesterol biosynthesis.

[00112] Because of the limited existing options for treating both AD and stroke, new therapeutics that can selectively activate only the PKC isoforms that elicit neuroprotection are needed.

PUFAs and MUFAs and Disease

[00113] A growing number of studies have suggested that omega-3 PUFAs can be beneficial for other mood disturbance disorders such as clinical depression, bipolar disorder, personality disorders, schizophrenia, and attention deficit disorders. Ross et al., *Lipids Health Dis.* 2007; 18;6:21. There is an abundance of evidence linking omega-3 fatty acids, particularly docosahexaenoic and eicosapentaenoic acids, and a healthy balance of omega-3 to omega-6 fatty acids, to lowering the risk of depression. Logan et al., *Lipids Health Dis.* 2004; 3: 25. Levels of omega-3 fatty acids were found to be measurably low and the ratio of omega-6 to omega-3 fatty acids were particularly high in a clinical study of patients hospitalized for depression. A recent study demonstrated that there was a selective deficit in docosahexaenoic in the orbitofrontal cortex of patients with major depressive disorder. McNamara et al. *Biol Psychiatry.* 2007;62(1):17-24. Several studies have also shown that subjects with bipolar disorder have lower levels omega-3 fatty acids. In several recent studies, omega-3 fatty acids were shown to be more effective than placebo for depression in both adults and children with bipolar

depression. Osher and Belmaker, *CNS Neurosci Ther.* 2009;15(2):128-33; Turnbull et al., *Arch Psychiatr Nurs.* 2008;22(5):305-11.

[00114] Extensive research also indicates that omega-3 fatty acids reduce inflammation and help prevent risk factors associated with chronic diseases such as heart disease, cancer, inflammatory bowel disease and rheumatoid arthritis. Calder et al., *Biofactors.* 2009;35(3):266-72; Psota et al., *Am J Cardiol.* 2006;98(4A):3i-18i; Wendel et al., *Anticancer Agents Med Chem.* 2009;9(4):457-70.

[00115] Monounsaturated fatty acids also have been shown to be beneficial in disorders. There is good scientific support for MUFA diets as an alternative to low-fat diets for medical nutrition therapy in Type 2 diabetes. Ros, American *Journal of Clinical Nutrition.* 2003; 78(3): 617S-625S. High-monounsaturated fatty acid diets lower both plasma cholesterol and triacylglycerol concentrations. Kris-Etherton et al., *Am J Clin Nutr.* 1999 Dec;70(6):1009-15.

[00116] The present invention includes use of cyclopropanated and epoxidized derivatives of PUFAs or MUFAs in which one, some, or all of the double bonds are replaced by a cyclopropane group or an epoxide group. The terminal function may be a free carboxylic acid, or a methyl ester, ethyl ester, or some other alkyl ester with an aliphatic or aromatic alcohol. This alcohol specifically may also include glycerol and derivatives thereof. Glycerol derivatives are biologically important because the fatty acids are most frequently found conjugated to glycerol in the form of phosphatidylcholine, phosphatidylserine, or phosphatidic acids. For example, triacylglycerols are compounds in which the carboxyl groups of fatty acids are esterified

to the hydroxyls of all three carbons found in glycerol are referred to as triacylglycerols or triglycerides.

[00117] The purpose of esterifying the carboxylic acid is to facilitate transport across the blood-brain barrier by eliminating the negative charge. The purpose of an alcohol group is also to facilitate transport across the blood-brain barrier.

[00118] In one embodiment, the fatty acid which forms the basis for the compounds used in the present invention is a polyunsaturated fatty acid having the following structure:

$$CH_3(CH_2)_4(CH{=}CHCH_2)\mathbf{x}(CH_2)\mathbf{y}COOH$$

[00119] wherein X is between 2 and 6, and Y is between 2 and 6, and include methylene- or polymethylene-interrupted polyenes. Exemplary polyunsaturated fatty acids include linoleic acid, γ-linoleic, arachidonic acid, and adrenic acid having the following structures:

Linoleic	$CH_3(CH_2)_4(CH{=}\ CHCH_2)_2(CH_2)_6COOH$
γ -Linolenic	$CH_3(CH_2)_4(CH{=}\ CHCH_2)_3(CH_2)_3COOH$
Arachidonic	$CH_3(CH_2)_4(CH{=}\ CHCH_2)_4(CH_2)_2COOH$
Adrenic	$CH_3(CH_2)_4(CH{=}\ CHCH_2)_4(CH_2)_4COOH$

These are omega-6 PUFAs.

[00120] In another embodiment, the fatty acid which forms the basis for the compounds used in the present invention is a polyunsaturated fatty acid having the following structure:

$$CH_3CH_2(CH{=}\ CHCH_2)\mathbf{x}(CH_2)\mathbf{y}COOH$$

[00121] wherein X is between 2 and 6, and Y is between 2 and 6 and include methylene- or polymethylene-interrupted polyenes. Exemplary polyunsaturated

fatty acids include α-lineoleic acid, docosahexaenoic acid, eicosapentaenoic acid, eicosatetraenoic acid having the following structures:

Alpha-Linolenic $CH_3CH_2(CH= CHCH_2)_3(CH_2)_6COOH$

Eicosatetraenoic $CH_3CH_2(CH=CHCH_2)_4(CH_2)_5COOH$

Eicosapentaenoic $CH_3CH_2(CH=CHCH_2)_5(CH_2)_2COOH$

Docosahexaenoic $CH_3CH_2(CH=CHCH_2)_6(CH_2)_2COOH$

These are known as omega-3 PUFAs.

[00122] In a specific embodiment, the compound of the present invention is an ester of a *cis*-PUFA, in which the hydroxyl group is replaced by an alkoxy group, and in which at least one of the double bonds has been cyclopropanated. The starting material for this embodiment has the following structures:

$CH_3(CH_2)_4(CH=CHCH_2)\mathbf{x}(CH_2)\mathbf{y}COOR$ or $CH_3CH_2(CH= CHCH_2)\mathbf{x}(CH_2)\mathbf{y}COOR$

[00123] wherein R is the alkyl group from an alcohol including monohydric alcohols and polyhydric alcohols including but not limited to methanol, ethanol, propanol, butanol, pentanol, glycerol, mannitol, and sorbitol.

[00124] In a further embodiment, the compound contains at least three cyclopropanated double bonds.

[00125] In another embodiment, the fatty acid which forms the basis for the compounds used in the present invention is a monounsaturated fatty acid having the following structure:

$$CH_3(CH_2)\mathbf{x}CH=CH(CH_2)\mathbf{y}COOH$$

wherein X and Y are odd numbers between 3 and 11.

[00126] Exemplary monounsaturated fatty acids that can be the basis for the compounds used in the present invention include *cis*- and trans- monounsaturated fatty acids such as oleic acid, elaidic acid, obtusilic acid, caproleic acid, lauroleic acid,

linderic acid, myristoleic acid, palmitoleic acid, vaccenic acid, gadoleic acid, erucic acid, and petroselinic acid.

[00127] An ester according to the invention, means a monoester or a polyester. Esters of fatty acids include methyl, propyl, and butyl esters, and also esters resulting from more complex alcohols such as propylene glycol. In non-limiting embodiments, R' is straight or branched and includes methyl, ethyl, propyl, isopropyl, butyl, isobutyl, secbutyl, tert-butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl, tridecyl, and tetradecyl. An ester may also be formed from a fatty acid linked to a fatty alcohol in an ester linkage.

[00128] The ester can be a alcohol ester, including but not limited to an aliphatic alcohol ester. In one embodiment, the alcohol ester is a glycerol ester. Glycerol esters of fatty acids include glycerol fatty acid ester, glycerol acetic acid fatty acid ester, glycerol lactic acid fatty acid ester, glycerol citric acid fatty acid ester, glycerol succinic acid fatty acid ester, glycerol diacetyl tartaric acid fatty acid ester, glycerol acetic acid ester, polyglycerol fatty acid ester, and polyglycerol condensed ricinoleic acid ester.

[00129] In another specific embodiment, the compound is an alcohol of a *cis*-PUFA in which at least one of the double bonds has been cyclopropanated. In a further embodiment, the compound is an alcohol of a *cis*-PUFA which contains at least three cyclopropanated double bonds. These compounds include but are not limited to linoleic alcohol dicyclopropane (**BR-105**), or linolenic alcohol tricyclopropane (**BR-104**). In this embodiment, R' can be a normal or branched chain alcohol or a phenolic alcohol.

[00130] In another embodiment, the compound of the present invention, the compound is a *cis*-polyunsaturated fatty acid, or derivative thereof, in which at least one

of the double bonds is replaced with an epoxyl group. In a further embodiment, the compound contains at least three epoxidized double bonds.

[00131] In a specific embodiment, the compound is an epoxidized ester of a *cis*-PUFA, including but not limited to a fatty alcohol ester. The esters can be the same esters as described above for the cyclopropanated PUFAS. In a further embodiment the alcohol can be an aliphatic alcohol ester, such as glycerol.

[00132] In another specific embodiment, the compound is an epoxidized *cis*-polyunsaturated fatty alcohol such as linoleic alcohol dicyclopropane or linolenic alcohol tricyclopropane. The alcohols can be the same as described above for the cyclopropanated PUFAS.

[00133] In another embodiment, the compound includes cyclopropanated or epoxidized lipids derived from *cis*-monounsaturated fatty acids (*cis*-monoenoic acids), such as oleic acid, elaidic acid, elaidic alcohol, oleyl alcohol, and 1-monolinoleyl rac-glycerol. Exemplary compounds include eliadic alcohol cyclopropane (**BR-106**), eliadic acid cyclopropane (**BR-107**), and oleyl alcohol cyclopropane (**BR-108**).

[00134] A further embodiment includes cyclopropanated lipids derived from *cis*-monounsaturated fatty acids or unsaturated fatty acids, fatty acid esters, or fatty acid alcohols, containing one or more epoxide residues, such as vernolic acid methyl ester cyclopropane (*e.g.*, **BR-109**).

[00135] In specific embodiments, the PUFAs which forms the basis of the cyclopropanated compounds used in the present invention include but are not limited to arachidonic acid (AA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA). Exemplary compounds for use in the method of the present invention include

docahexaenonic acid methyl ester hexacyclopropane (**BR-111**); eicosapentaenoic acid methyl ester pentacyclopropane (**BR-114**); and arachidonic acid methyl ester tetracyclopropane (**BR-115**).

[00136] In a further specific embodiment, the compound is a cyclopropanated PUFA derivative of docosahexaenoic acid having the following structure:

[00137] in which R is H or an alkyl group. In a specific embodiment, R is CH3 (BR-111 or DHA-CB6 methyl ester or methyl-3-(2-((2-((2-((2-((2-((2-ethylcyclopropyl)methyl)cyclopropyl)methyl)cyclopropyl)methyl)cyclopropyl)methyl)cyclopropyl)methyl)cyclopropyl)propanoate.

[00138] In another specific embodiment, the PUFA derivative has the following structure:

[00139] This compound is BR-114 (EPA-CP5 or methyl 4-(2((2-((2-((2-ethylcyclopropyl)methyl)cyclopropyl)methyl)cyclopropyl)methyl)cyclopropyl)methyl)cyclopropyl)butanoate methyl ester).

[00140] In still another specific embodiment, the PUFA derivative has the following structure:

[00141] This compound is BR-115 (AA-CP4 or methyl 4-(2-((2-((2-((-pentylcyclopropyl)methyl)cyclopropyl)methyl)cyclopropyl)methyl)cyclopropyl)butanoate methyl ester).

[00142] In yet another specific embodiment, the PUFA derivative has the following structure:

in which R is H or an alkyl ester. In a specific embodiment, R is CH3.

[00143] Naturally cyclopropanated or epoxidized MUFAS or ester or alcohol derivatives thereof contemplated for use in the present invention include malvenic acid, vernolic acid, and sterculic acid. An exemplary compound is vernolic acid methyl ester (BR-117).

Methods of synthesis

[00144] Fatty acids, and esters and alcohols thereof, can be obtained or made from purification from natural sources, *e.g.*, fish oil, flaxseed oil, soybeans, rapeseed oil, or algae, or synthesized using a combination of microbial enzymatic synthesis and chemical synthesis. As one example, fatty acid methyl esters can be produced by the transesterification of triglycerides of refined/edible type oils using methanol and an homogeneous alkaline catalyst.

[00145] Methods of cyclopropanation of double bonds in hydrocarbons are well known. As one example, the modified Simmons-Smith reaction is a standard method for converting double bonds to cyclopropanes. Tanaka and Nishizaki, *Bioorg. Med. Chem. Let.* 2003; 13: 1037-1040; Kawabata and Nishimura, *J. Tetrahedron.* 1967;

24: 53-58; and Denmark and Edwards, *J. Org Chem.* 1991; 56: 6974. In this reaction, treatment of alkenes with metal carbenoids, *e.g.*, methylene iodide and diethylzinc, result in cyclopropanation of the alkene. See also, Ito *et al.*, Organic Syntheses. 1988; 6:327. Cyclopropanation of methyl esters of was also effected using diazomethane in the presence of palladium (II) acetate as catalyst. Gangadhar et al., *Journal of the American Oil Chemists' Society.* 1988; 65(4): 601-606.

[00146] Methods of epoxidation are also well known and typically involve reaction of fatty acids dioxiranes in organic solvents. Sonnet et al., *Journal of the American Oil Chemists' Society.* 1995; 72(2):199-204. As one example, epoxidation of PUFA double bonds can be achieved using dimethyldioxirane (DMD) as the epoxidizing agent. Grabovskiy et al., *Helvetica Chimica Acta.* 2006; 89(10): 2243-53.

Methods of treatment

[00147] The present invention contemplates treatment of neurological diseases associated with pathogenic Aβ such as AD and stroke using the PUFA derivatives disclosed herein. The present invention also contemplates prevention of neurological diseases associated with pathogenic Aβ using the PUFA derivatives disclosed herein. Without being limited to any particular mechanism, selective activation of PKCε may result in increased activation of TACE, with a concomitant decrease in production of Aβ. However, this appears to occur mainly in non-neuronal cells such as fibroblasts. Activation of PKCε may also reduce the hyperphosphorylation of the pathogenic tau protein in AD. Activation of PKCε may also induce synaptogenesis or prevent apoptosis in AD or following stroke. Activation of PKCε may also protect rat neurons from Aβ-mediated neurotoxicity through inhibition of GSK-3β. PKCε activators

may also counteract the effect of Aβ on the downregulation of PKC α/ε, and thereby reverse or prevent the Aβ-induced changes. Another possible mechanism of action is the activation of Aβ-degrading enzymes such as endothelin-converting enzyme. The results of experiments presented in the Examples suggest that this may be the mechanism of action.

[00148] Yet another mechanism may be by stimulation of PKC-coupled M1 and M3 muscarinic receptors, which is reported to increase nonamyloidogenic APP processing by TACE. Rossner et al., *Prog. Neurobiol.* 1998; 56: 541-569. Muscarinic agonists rescue 3x-transgenic AD mice from cognitive deficits and reduce Aβ and tau pathologies, in part by activating the TACE/ADAM17 nonamyloidogenic pathway. Caccamo et al., *Neuron.* 2006; 49:671-682. Muscarinic receptor signaling is closely tied to PKC. Muscarinic receptor mRNA is regulated by PKC and neuronal differentiation produced by M1 muscarinic receptor activation is mediated by PKC. Barnes et al., *Life Sci.* 1997; 60:1015-1021; Vandemark et al., *J. Pharmacol. Exp. Ther.* 2009; 329(2): 532-42.

[00149] Other disorders contemplated for treatment by the methods of the present invention include, mood disorders such as depressive disorders and bipolar disorder, schizophrenia, rheumatoid arthritis, cancer, cardiovascular disease, type 2 diabetes, and any other disorder in which PUFAs or MUFAs have been shown to be beneficial, including but not limited to those mention in the background.

Formulation and administration

[00150] The PUFA derivatives may be produced in useful dosage units for administration by any route that will permit them to cross the blood-brain barrier. It has

been demonstrated PUFAs from plasma are able to cross into the brain. Rapoport et al., *J. Lipid Res.* 2001. 42: 678–685. Exemplary routes include oral, parenteral, transmucosal, intranasal, inhalation, or transdermal routes. Parenteral routes include intravenous, intra-arteriolar, intramuscular, intradermal, subcutaneous, intraperitoneal, intraventricular, intrathecal, and intracranial administration.

[00151] The compounds of the present invention can be formulated according to conventional methods. The PUFA derivative compounds can be provided to a subject in standard formulations, and may include any pharmaceutically acceptable additives, such as excipients, lubricants, diluents, flavorants, colorants, buffers, and disintegrants. Standard formulations are well known in the art. See *e.g.*, Remington's Pharmaceutical Sciences, 20th edition, Mack Publishing Company, 2000.

[00152] In one embodiment, the compound is formulated in a solid oral dosage form. For oral administration, *e.g.*, for PUFA, the pharmaceutical composition may take the form of a tablet or capsule prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (*e.g.*, pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (*e.g.*, lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (*e.g.*, magnesium stearate, talc or silica); disintegrants (*e.g.*, potato starch or sodium starch glycolate); or wetting agents (*e.g.*, sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as

suspending agents (*e.g.*, sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (*e.g.*, lecithin or acacia); non-aqueous vehicles (*e.g.*, almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (*e.g.*, methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

[00153] As one example, the drug Omacor® contains concentrated combinations of ethyl esters of an omega-3 PUFAS. Each 1-g capsule contains at least 900 mg of the ethyl esters of omega-3 fatty acids, primarily EPA (465 mg) and DHA (375 mg), according to the drug's label. Omacor® is administered up to 4 times per day as 1-gram transparent soft gelatin capsules filled with light-yellow oil. A similar composition can be used to administer the PUFA compounds of the present invention, although the present invention contemplates use of a lower dose of the PUFA derivatives. Stable wax-ester formulations of PUFAs have also been described by transesterification of stoichiometric amounts of ethyl esters enriched with n-3 PUFA and long-chain alcohols (18-22 carbon atoms) by transesterification of stoichiometric amounts of ethyl esters enriched with n-3 PUFA and long-chain alcohols (18–22 carbon atoms). Goretta et al., *Lebensmittel-Wissenschaft und-Technologie*. 2002; 35(5): 458-65.

[00154] In another embodiment, the PUFA compound is formulated for parenteral administration. The compound may be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as

suspensions, solutions, dispersions, or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

[00155] In a specific embodiment, the PUFA derivatives of the present invention are administered with a hydrophobic carrier. Hydrophobic carriers include inclusion complexes, dispersions (such as micelles, microemulsions, and emulsions), and liposomes. Exemplary hydrophobic carriers are inclusion complexes, micelles, and liposomes. These formulations are known in the art (Remington's: The Science and Practice of Pharmacy 20th ed., ed. Gennaro, Lippincott: Philadelphia, PA 2003). The PUFA derivatives of the present invention may be incorporated into hydrophobic carriers, for example as at least 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, or 90% of the total carrier by weight. In addition, other compounds may be included either in the hydrophobic carrier or the solution, *e.g.*, to stabilize the formulation.

[00156] In addition to the formulations described previously, the PUFA derivative may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

[00157] In another embodiment, the PUFA derivative can be delivered in a vesicle, particularly a micelle, liposome or an artificial LDL particle as described in U.S. patent application serial no. 11/648,808 to Alkon et al.

[00158] The doses for administration may suitably be prepared so as to deliver from 1 mg to 10 g, preferably from 10 mg to 1 g and very preferably from 250 mg to 500 mg of the compound per day. When prepared for topical administration or parenteral formulations they may be made in formulae containing from 0.01% to 60% by weight of the final formulation, preferably from 0.1% to 30% by weight, and very preferably from 1% to 10% by weight. The optimal daily dose will be determined by methods known in the art and will be influenced by factors such as the age of the patient and other clinically relevant factors.

Combination Drug Therapy

[00159] The PUFA compound can be used to treat patients with AD or other neurological disorders associated with Aβ in combination with other drugs that are also used to treat the disorder. Exemplary non-limiting pharmacological agents approved in the United States for the treatment of AD include cholinesterase inhibitors such as Aricept® (donepezil), Exelon® (rivastigmine), Reminyl® (galantamine), and NMDA receptor antagonists such as Namenda® (memantine). Other potential therapeutic agents include protease inhibitors (see *e.g.*, U.S. Patent Nos. 5,863,902; 5,872,101; inhibitors of Aβ production such as described in U.S. Patent Nos. 7,011,901; 6,495,540; 6,610,734; 6,632,812; 6,713,476; and 6,737,420; modulators of Aβ aggregation, described in 6,303,567; 6,689,752; and inhibitors of BACE such as disclosed in U.S. Patent Nos. 6,982,264; 7,034,182; 7,030,239. Exemplary drugs used for the treatment of stroke include aspirin, anti-platelet medications such as tissue plasminogen activator or other anticoagulants.

[00160] In a particular embodiment, the present invention contemplates combination therapy with other PKC activators, including but not limited to benzolactam macrocyclic lactones. Bryostatin-1 is a macrocyclic lactone that has been shown to modulate PKC and result in an increase in cleavage of APP by TACE into the non-amyloidogenic pathway. Bryostatin was able to increase the duration of memory retention of the marine slug *Hermissenda crassicornis* by over 500%, and was able to dramatically increase the rate of learning in rats. See U.S. patent application 10/919,110; Kurzirian et al., *Biological Bulletin.* 2006; 210(3): 201-14; Sun and Alkon, *European Journal of Pharmacology.* 2005;512(1): 43-51. Other non-limiting PKC activators are described in pending U.S. patent application serial number 12/068,742 to Alkon et al.

[00161] Combinations with drugs that indirectly increase TACE, such as by inhibiting endogenous TACE inhibitors or by increasing endogenous TACE activators. An alternative approach to activating PKC directly is to increase the levels of the endogenous activator, diacylglycerol. Diacylglycerol kinase inhibitors such as 6-(2-(4-[(4-fluorophenyl)phenylmethylene]-1-piperidinyl)ethyl)-7-methyl-5H-thiazolo[3,2-a]pyrimidin-5-one (R59022) and [3-[2-[4-(bis-(4-fluorophenyl)methylene]piperidin-1-yl)ethyl]-2,3-dihydro-2-thioxo-4(1H)-quinazolinone (R59949) enhance the levels of the endogenous ligand diacylglycerol, thereby producing activation of PKC. Meinhardt et al. (2002) *Anti-Cancer Drugs* 13: 725.

[00162] Still another embodiment is combination therapy with BACE inhibitors. BACE inhibitors are known and include CTS-21166, owned by CoMentis Inc., which has shown positive results in a human clinical trial. Other BACE inhibitors

are described in published International PCT application WO2007/019080 and in Baxter et al., *Med. Chem.* 2007; 50(18): 4261-4264.

[00163] Compounds used in combination therapy can be administered in the same formulation as the PUFA compound of the present invention, where compatible, or can be administered in separate formulations.

Evaluation of Treatment

[00164] Evaluation of treatment with the PUFA derivatives of the present invention can be made by evaluation improvement in symptoms or clinical surrogate markers of the disease. For example, improvement in memory or cognitive skills in a treated AD subject may suggest that there is a reduction of pathogenic Aβ accumulation. Examples of cognitive phenotypes include, but are not limited to, amnesia, aphasia, apraxia and agnosia. Examples of psychiatric symptoms include, but are not limited to, personality changes, depression, hallucinations and delusions. As one non-limiting example, the Diagnostic and Statistical Manual of Mental disorders, 4th Edition (DSM-IV-TR) (published by the American Psychiatric Association) contains criteria for dementia of the Alzheimer's type.

[00165] Phenotypic manifestations of AD may also be physical, such as by the direct (imaging) or indirect (biochemical) detection of Aβ plaques. In vivo imaging of Aβ can be achieved using radioiodinated flavone derivatives as imaging agents (Ono et al., *J Med Chem.* 2005;48(23):7253-60) and with amyloid binding dyes such as putrescine conjugated to a 40-residue radioiodinated A peptide (yielding 125I-PUT-A 1−40), which was shown to cross the blood-brain barrier and bind to Aβ plaques. Wengenack et al., *Nature Biotechnology.* 2000; 18(8): 868-72. Imaging of Aβ also was

shown using stilbene [11C]SB-13 and the benzothiazole [11C]6-OH-BTA-1 (also known as [11C]PIB). Verhoeff et al., *Am J Geriatr Psychiatry*. 2004; 12:584-595.

[00166] Quantitation of Aβ (1-40) in the peripheral blood has been demonstrated using high-performance liquid chromatography coupled with tandem mass spectrometry in a linear ion trap. Du et al., *J Biomol Tech*. 2005;16(4):356-63. Detection of single Aβ protein aggregates in the cerebrospinal fluid of Alzheimer's patients by fluorescence correlation spectroscopy also has been described. Pitschke et al., *Nature Medicine*. 1998; 4: 832-834. U.S. Patent 5,593,846 describes a method for detecting soluble Aβ. Indirect detection of Aβ peptide and receptor for advanced glycation end products (RAGE) using antibodies also has been described. Lastly, biochemical detection of increased BACE-1 activity in cerebrospinal fluid using chromogenic substrates also has been postulated as diagnostic or prognostic indicator of AD. Verheijen et al., *Clin Chem*. 2006; 52:1168-1174.

[00167] Current measures for evaluation AD include observation of a clinical core of early, progressive and significant episodic memory loss plus one or more abnormal biomarkers (biological indicators) characteristic of AD, including atrophy (wasting) of the temporal lobe as shown on MRI; abnormal Aβ protein concentrations in the cerebrospinal fluid; a specific pattern showing reduced glucose metabolism on PET scans of the brain; and a genetic mutation associated with within the immediate family.

EXAMPLES

Example 1: Patient population and Cell culture

[00168] Human dermal fibroblasts from Alzheimer's disease patients (AD), non-AD dementia (Huntington's disease, Parkinson's disease and Frontotemporal dementia) patients, and age-matched control (AC) cases were obtained from the Coriell Institute of Medical Research (Camden, NJ). Fibroblast cells were maintained in DMEM with low glucose (Invitrogen, USA) supplemented with 10% FBS, and were grown to 100% confluence before experiments. Ten different examples of AD patients (four familial type and six sporadic; among these nine out of ten were autopsy confirmed), eleven AC and eight Huntington's disease, one Parkinson's disease and one Front temporal dementia were considered for the study (Table: 1). The average age of the AD cases was 69.6 ± 13.01 (SD) yrs, AC cases was 63.364 ± 7.65 (SD) yrs and non-AD dementia cases were 56.44 ± 9.7 (SD) yrs.

[00169] **Protein isolation:** Flasks containing cells were washed 3× with 1X PBS (pH 7.4) and the cells were collected using a cell scraper. The collected cells were transferred to 1.5 ml microcentrifuge tubes and centrifuged at 1000 rpm for 5 mins. The Cell pellet obtained was suspended in homogenizing buffer (10mM Tris pH 7.4, 1mM PMSF, 10mM EGTA, 10mM EDTA and 50mM NaF) and sonicated for 30 secs. The homogenate was centrifuged again at 4°C for 10mins at 10000 rpm and the supernatant was collected and transferred to a new tube for protein estimation. Total protein concentration was measured using a Bradford Protein assay Kit (Thermo Scientific, USA).

[00170] **Immunoblot analysis:** Protein lysates (20 μg of protein each) were boiled in 2X Laemmli buffer for 10 min and separated using a 4-20% gradient Tris-Glycine gels. Separated proteins were transferred to nitrocellulose membrane and the membrane was blocked in BSA at room temperature (RT) for 15 min and incubated with 1:2000 dilution anti-PKC-ε rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA.; Cat No: sc-214), and 1:5000 dilution anti-β-tubulin, class III rabbit monoclonal antibody (Millipore Corporation, Billerica MA, Cat No: 04-1049) for 1 hr at RT. After the incubation, the membrane fractions were washed 3× with standard western blot washing buffer and further incubated with alkaline phosphatase conjugated secondary antibody (Jackson Immunoresearch Laboratories, USA) at 1:10000 dilution for 45 min. The membrane fractions were finally washed 3× with standard western blot washing buffer and developed using the 1-step NBT-BCIP substrate (Thermo Scientific, Rockford, IL). Signal intensities of the images were recorded in the ImageQuant RT-ECL (GE Life Sciences, Piscataway, NJ) and densitometric quantification was performed using the IMAL software (Blanchette Rockefeller Neurosciences Institute, Morgantown, WV). Intensities quantified in this way, for PKC-ε were normalized against β-tubulin for each lane.

[00171] **Immunofluorescence:** Fibroblasts cells were grown in chambered slides (Nunc, Rochester, NY) at low density. For immunofluorescence staining, the cells were washed 3 x with 1×PBS (pH 7.4) and fixed with 4% paraformaldehyde for 4 min. Following fixation, cells were blocked and permeabilized with 5% serum and 0.3% Triton X 100 in 1 x PBS for 30 min. Cells were washed 3× with 1 x PBS and incubated with rabbit polyclonal PKC-ε antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1

hr at 1:100 dilution. After the incubation slides were washed 3 x in 1 x PBS and were incubated with the FITC anti-rabbit IgG (Jackson Immunoresearch Laboratories, USA) for 1 hr at 1:400 dilution. Cells were washed and also stained with DAPI (4',6-diamidino-2'-phenylindole, dihydrochloride) (Thermo Scientific, USA). Finally, the slides were washed and mounted in glycerol PBS mounting solution and were viewed under the LSM 710 Meta confocal microscope (Zeiss, Germany) at 350 nm and 490 nm excitation and 470 nm and 525 nm emission for DAPI and FITC, respectively. Five individual fields were captured by 63 x oil lens magnification were analyzed for the mean fluorescence intensity (MFI) in each channel.

[00172] **RT-PCR:** RNA was isolated from ~1 X 10^6 cells using Trizol reagent (Invitrogen, USA) following manufacturer's protocol. Briefly, 2 μg of RNA was reverse transcribed using oligodT and Superscript III (Invitrogen, USA) at 50°C for 1 hr. Two μl of the cDNA product was amplified using primers for PKC-ε (Forward Primer – AGCCTCGTTCACGGTTCTATGC, Reverse primer – GCAGTGACCTTCTGCATCCAGA), and β-tubulin (Forward Primer – TTGGGAGGTGATCAGCGATGAG, Reverse primer – CTCCAGATCCACGAGCACGGC) (Origene, Rockville, MD). The amplicons were analysed in an E-Gel (Invitrogen, USA) following 25 cycle amplification at 55°C annealing temperature. The gel image was documented using a Fuji Image gel scanner (FLA-9000, Fuji Film) and densitometric quantification was performed using the IMAL software (Blanchette Rockefeller Neurosciences Institute, Morgantown, WV). Data were represented as normalized ratio of PKC-ε OD (Optical Density) against β-tubulin OD for three independent experiments.

[00173] Preparation of soluble oligomeric Aβ: Oligomeric Aβ was prepared following the method described by Noguchi et al., (2009). Aβ generated by this method was reported to be highly neurotoxic, 10-15nm spherical Aβ assemblies termed as amylospheroids (ASPDs). For synthesis of ASPDs, $A\beta_{1-42}$ (Anaspec, USA) was dissolved in 1,1,1,3,3,3-hexafluro-2-propanol at 100μM concentration overnight at 4°C and then at 37°C for 3 hrs. Finally, the dissolved Aβ was lyophilized in aliquots (40nmol/tube). The lyophilized Aβ was dissolved in 50% PBS at 1 μM concentration and slowly rotated at 4°C for 14hrs. Flowing incubation the toxic ASPDs were purified using the 100kDa molecular mass cutoff filters (Ultrafree-MC, Millipore, USA). The retentates with molecular weight of > 100kda were used for treating fibroblasts.

[00174] Treatment of skin fibroblasts with oligomeric $A\beta_{1-42}$: Skin fibroblasts both from AD and AC cases were cultured for 7 days to 100% confluence. The confluent cells were treated with 500nM (final concentration) of ASPDs for 24 hrs at 37°C after they were 100% confluent. Following incubation, the cells were washed 3× with 1×PBS (pH 7.4) and processed for western blotting as described earlier. The resulting band intensities for PKC-ε were quantified using ImageQuant RT-ECL (GE Life Sciences, USA) and densitometric analysis was performed using the IMAL software (Blanchette Rockefeller Neurosciences Institute, Morgantown, WV).

Example 2: Synthesis of Fatty Acid Methyl Esters Cyclopropanated Fatty Acid Methyl Esters.

[00175] Synthesis of cyclopropanated fatty acids. Methyl esters of polyunsaturated fatty acids were cyclopropanated using the modified Simmons-Smith

reaction using chloroiodomethane and diethylzinc (Tanaka et al., *Bioorg. Med. Chem.* Let. 2003; 13: 1037-40; Furukawa et al., *Tetrahedron.* 1967; 53-58; Denmark et al., *J. Org. Chem.* 1991; 56: 6974-81). All apparatus was baked at 60°C for 1 hr and dried using a flame with dry nitrogen. A 100 ml 3-neck round bottom flask with a stirring bar and a temperature probe was surrounded by an ice-dry ice mixture and filled with 1.25g (4.24 mmol) linoleic acid methyl ester or docosahexaenoic acid methyl ester in 25 ml dichloromethane and bubbled with N_2. A 1M solution of diethylzinc (51 ml, 54.94 mmol) in hexane was added anaerobically using a 24-inch-long 20-gauge needle and the solution was cooled to -5°C. Diiodomethane (8.2 ml, 101.88 mmol) or chloroiodomethane (ClCH2 I) was added dropwise, one drop per second, with constant stirring. The rate of addition was decreased if necessary to maintain the reaction mixture below 2°C. The reaction mixture became cloudy during the reaction and an insoluble white zinc product was liberated. The flask was sealed and the mixture was allowed to react for 1 hr and then allowed to come to room temperature gradually over 2 hr.

[00176] To prevent the formation of an explosive residue in the hood, diethylzinc was not evaporated off. The mixture was slowly poured into 100 ml of water under stirring to decompose any excess diethylzinc. Ethane was evolved. The mixture was centrifuged at 5000 rpm in glass centrifuge tubes and the upper aqueous layer discarded. The white precipitate was extracted with CH_2Cl_2 and combined with the organic phase. The organic phase was washed with water and centrifuged. The product was analyzed by silica gel G TLC using hexane plus 1% ethyl acetate and purified by chromatography on silica gel using increasing concentrations of 1-10% ethyl acetate in n-hexane and evaporated under nitrogen, leaving the methyl ester as a colorless oil.

[00177] The Simmons-Smith reaction preserves the stereochemistry of the starting materials. Furukawa et al., *Tetrahedron*. 1967; 53-58. Docosahexaenoic acid methyl ester was converted into DHA-CP6 in 90-95% yield. The product was a colorless oil with a single absorbance maximum at 202 nm in ethanol and no reaction with I_2. The IR spectrum showed cyclopropane ring absorption at 3070 and 1450 cm^{-1}. Under the same conditions, eicosapentaenoic acid methyl ester was converted to EPA-CP5, and arachidonic acid methyl ester was converted to AA-CP4. Linoleic acid methyl ester was converted to DCP-LA methyl ester which was identical to a known sample.

[00178] Hydrolysis of methyl ester. The methyl ester (0.15 g) was dissolved in 1 ml 1N LiOH and 1 ml dioxane. Dioxane and methanol were added until it became homogeneous and the solution was stirred 60° overnight. The product was extracted in CH_2Cl_2 and centrifuged. The aqueous layer and white interface were re-extracted with water and washed until the white layer no longer formed. The product was evaporated under N_2 and purified by chromatography on silica gel. The product, a colorless oil, eluted in 20% EtOAc in n-hexane. Its purity was checked by TLC in 10% EtOAc/hexane and by C18 RP-HPLC using UV detection at 205 nm.

[00179] The epoxide groups can be introduced by conventional means, *e.g.*, by oxidation of the appropriate alkene with m-chloroperbenzoic acid or t-butylhydroperoxide.

[00180] Other compounds synthesized include those depicted in Figure 1 (**BR-101** through **BR-118**).

Example 2: Activation of Purified PKC Epsilon using Docosahaexanoic Acid

[00181] Protein kinase C assay. Recombinant PKC (1 ng of alpha or epsilon isoform) was mixed with the **BR-101** (DCP-LA) in the presence of 10 micromolar histones, 5 mM $CaCl_2$, 1.2 μg/μl phosphatidyl-L-serine, 0.18 μg/μl 1,2-dioctanoyl-sn-glycerol (DAG), 10 mM $MgCl_2$, 20 mM HEPES (pH 7.4), 0.8 mM EDTA, 4 mM EGTA, 4% glycerol, 8 μg/ml aprotinin, 8 μg/ml leupeptin, and 2 mM benzamidine. 0.5 micro Ci [$^{\gamma 32}$P]ATP was added. The incubation mixture was incubated for 15 min at 37 degrees in a total volume of 10 microliters. The reaction was stopped by spotting the reaction mixtures on 1x2 cm strips of cellulose phosphate paper (Whatman P81) and immediately washing twice for 1 hr in 0.5% H_3PO_4. The cellulose phosphate strips were counted in a scintillation counter. In some experiments, phosphatidylserine, diacylglycerol, and/or calcium were removed.

[00182] DHA methyl ester was purchased from Cayman Chemical (Ann Arbor, ME). PKC isozymes were from Calbiochem (San Diego, CA). Purified PKCε was purchased from Calbiochem.

Results

[00183] PKC measurements using purified PKCε showed that, at the lowest concentration tested (10 nM), compound **BR-101** produced a 2.75-fold activation of PKCε. PKCα was not affected (data not shown). Compound **BR-102** also selectively elicited activation of PKCε to about 1.75 fold over unactivated PKCε. The effectiveness of these compounds in activating PKCε at low concentrations suggests that they will be good therapeutic candidates.

Example 3: Activation of Purified or Cellular PKC Epsilon using Other PKC Activators

[00184] Materials. Culture media were obtained from K-D Medical (Columbia, MD) or Invitrogen (Carlsbad, CA). Aβ1-42 was purchased from Anaspec (San Jose, CA). Polyunsaturated fatty acid methyl esters were obtained from Cayman Chemicals, Ann Arbor, MI. Other chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). PKC isozymes were from Calbiochem (San Diego, CA). Purified PKCε was purchased from Calbiochem.

[00185] Cell culture. Rat hippocampal H19-7/IGF-IR cells (ATCC, Manassas, VA) were plated onto poly-L-lysine coated plates and grown at 35°C in DMEM/ 10% FCS for several days until about 50% coverage was obtained. The cells were then induced to differentiate into a neuronal phenotype by replacing the medium with 5 ml N_2 medium containing 10 ng/ml basic fibroblast growth factor at 39°C and grown in T-75 flasks at 37°C. Human SH-SY5Y neuroblastoma cells (ATCC) were cultured in 45% F12K / 45% MEM / 10% FCS. Mouse N2A neuroblastoma cells were cultured in DMEM/ 10% FCS without glutamine. Rat hippocampal neurons from 18-day-old embryonic

[00186] Sprague Dawley rat brains were plated on 12- or 96-well plates coated with poly-D-lysine (Sigma-Aldrich, St. Louis, MO) in B-27 neurobasal medium containing 0.5 mM glutamine and 25 μM glutamate (Invitrogen, Carlsbad, CA) and cultured for three days in the medium without glutamate. The neuronal cells were grown under 5% CO_2 in an incubator maintained at 37 °C for 14 days.

[00187] All experiments on cultured cells were carried out in triplicate unless otherwise stated. All data points are displayed as mean ±SE. **BR-101** (DCP-LA)

was used as its free acid in all experiments, while **BR-111** (DHA-CP6), **BR-114** (EPA-CP5), and **BR-116** (AA-CP4) were used as their methyl esters.

[00188] Protein kinase C assay. Rat hippocampal cells were cultured and scraped in 0.2 ml homogenization buffer (20 mM Tris-HCl, pH 7.4, 50 mM NaF, 1 μg/ml leupeptin, and 0.1 mM PMSF) and homogenized by sonication in a Marsonix micro-probe sonicator (5 sec, 10W). To measure PKC, 10 μl of cell homogenate or purified PKC isozyme (purchased from Calbiochem) was incubated for 15 min at 37°C in the presence of 10μM histones, 4.89 mM $CaCl_2$, 1.2μg/μl phosphatidyl-L-serine, 0.18μg/μl 1,2-dioctanoyl-sn-glycerol, 10 mM $MgCl_2$, 20 mM HEPES (pH 7.4), 0.8 mM EDTA, 4 mM EGTA, 4% glycerol, 8 μg/ml aprotinin, 8 μg/ml leupeptin, and 2 mM benzamidine. 0.5μCi [$^{\gamma-32}$P]ATP was added and ^{32}P-phosphoprotein formation was measured by adsorption onto phosphocellulose as described previously. Nelson and *Alkon, J. Neurochemistry.* 1995; 65: 2350-57. For measurements of activation by **BR-101** (DCP-LA) and similar compounds, PKC activity was measured in the absence of diacylglycerol and phosphatidylserine, as described by Kanno et al., and PKC δ, ε, η, and μ were measured in the absence of added EGTA and CaCl2 , as described by Kanno et al., *J. Lipid Res.* 2006; 47: 1146-50. Low concentrations of Ca^{2+} are used because high Ca^{2+} interacts with the PKC phosphatidylserine binding site and prevents activation. For measurements of bryostatin activation, 1,2-diacylglycerol was omitted unless otherwise stated.

Results and Discussion

[00189] To determine their PKC isozyme specificity, the new compounds were preincubated with purified PKC for five minutes and the PKC activity was

measured radiometrically. As shown for Example, 2, above, **BR-101** (DCP-LA) was an effective activator of PKCε at 10 μM but had relatively small effects on the other PKC isoforms (data not shown). At higher concentrations **BR-101** (DCP-LA) partially inhibited PKCδ (about 1-100 μM) and activated PKCγ (50-100 μM) (data not shown).

[00190] **BR-111** (DHA-CP6), **BR-114** (EPA-CP5), and **BR-115** (AA-CP4), which are cyclopropanated derivatives of docosahexaenoic acid, eicosapentaenoic acid, and arachidonic acid, respectively, activated purified PKCε to a similar extent. The concentration needed to activate PKC was approx. 100 times lower than for **BR-101** (DCP-LA), suggesting higher affinity. Cyclopropanated linolenyl and linoleyl alcohols (**BR-104** and **BR-105**), epoxystearic acid (**BR-116**), and vernolic acid methyl ester (**BR-117**) had little or no effect on PKC. Cyclopropanated vernolic acid methyl ester (**BR-109**) inhibited PKCε at concentrations above 1μM.

[00191] PKC activators that bind to the diacylglycerol binding site, including bryostatin, gnidimacrin, and phorbol esters, produce a transient activation of PKC activity, followed by a prolonged downregulation. Nelson et al., *Trends in Biochem. Sci.* 2009; 34: 136-45. This was confirmed in cultured rat hippocampal cells. Incubation of rat H19-7/IGF-IR cells with (0.04 nM and 0.2 nM) bryostatin produced a 2-fold activation that lasted 30 min, followed by a 20% downregulation that returned to baseline by 24h (data not shown). In contrast, PKC exposed to DCP-LA remained elevated for at least four hours. This sustained activation was only observed in primary neurons.

[00192] Even though bryostatin has a higher affinity for PKC than phorbol 12-myristate 13-acetate (PMA)(EC50 = 1.35 nM vs. 10 nM), bryostatin was

much less effective than PMA at downregulating PKC. PKC activity is strongly downregulated by phorbol ester at 8h, while PKC in bryostatin-treated cells is at or near the baseline (data not shown). This difference may explain the increases in Aβ produced by PdBu reported by da Cruz e Silva et al. *J. Neurochem.* 2009: 108: 319-30. These investigators applied 1μM PdBu to cultured COS cells for 8h and observed an increase in Aβ. This increase was attributed to downregulation of PKC by the phorbol ester, which is consistent with these results. Downregulation could not be measured for DCP-LA and related compounds.

Example 4: Effects of PKC Activators on Aβ Production and Degradation

[00193] Cell culture. Cell culture was performed as described above for Example 3.

[00194] Aβ Measurement and Cell Viability Assay. Aβ was measured using an Aβ 1-42 human fluorimetric ELISA kit (Invitrogen) according to the manufacturer's instructions. Results were measured in a Biotek Synergy HT microplate reader. AlamarBlue and CyQuant NF (Invitrogen) according to the manufacturer's instructions.

Results and Discussion

[00195] To measure the effects of PKCε activation on Aβ production, we used mouse neuro2a (N2a) neuroblastoma cells transfected with human APPSwe/PS1D, which produce large quantities of Aβ. Petanceska et al., *J. Neurochem.* 1996; 74: 1878-84. Incubation of these cells for 24h with various concentrations of PKC activators. bryostatin, **BR-101** (DCP-LA) and **BR-111** (DHA-CP6) markedly reduced the levels of both intracellular and secreted Aβ. With bryostatin, which activates PKC by binding to

the diacylglycerol-binding site, the inhibition was biphasic, with concentrations of 20 nM or higher producing no net effect. This may be explained by the ability of this class of PKC activators to downregulate PKC when used at high concentrations. In contrast, **BR-101** (DCP-LA) and **BR-111** (DHA-CP6), which bind to PKC's phosphatidylserine site, showed monotonically increasing inhibition at concentrations up to 10 to 100 μM with no evidence of downregulation at higher concentrations.

[00196] To determine whether the reduced levels of Aβ caused by PKC activators were due to inhibition of Aβ synthesis or activation of Aβ degradation, we applied **BR-111** (DHA-CP6) (0.01 to 10 μM) and low concentrations (100 nM) of exogenous monomeric Aβ-42 to cultured SH-SY5Y cells. This concentration of Aβ is too low to produce measurable toxicity or cell death. Since SH-SY5Y cells produce only trace amounts of Aβ, this experiment was an effective test of the ability of PKC activators to enhance Aβ degradation. By 24h, most of the Aβ had been taken up by the cells and the concentration of Aβ in the culture medium was undetectable. Addition of 0.01 to 10μM DHA-CP6 to the cells reduced the cellular levels of Aβ by 45-63%, indicating that the PKCε activator increased the rate of degradation of exogenous Aβ.

[00197] DHA-CP6, bryostatin, and DCP-LA had no effect on cell survival or on proliferation as measured by alamar Blue and CyQuant staining, indicating that the reduction in Aβ production did not result from cell proliferation or a change in cell survival.

Example 5: Effects of PKC Activators on TACE Activity

[00198] TACE Assay. TACE was measured by incubating 5 μl cell homogenate, 3 μl buffer (50 mM Tris-HCl 7.4 plus 25 mM NaCl plus 4% glycerol), and

1 μl of 100 μM TACE substrate IV (Aβz-LAQAVRSSSR-DPa) (Calbiochem) for 20 min at 37° in 1.5-ml polypropylene centrifuge tubes(Jin et al., *Anal. Biochem.* 2002; 302: 269-75). The reaction was stopped by cooling to 4°C. The samples were diluted to 1 ml and the fluorescence was rapidly measured (ex = 320 nm, em = 420 nm) in a Spex Fluorolog 2 spectrofluorometer.

Results and Discussion

[00199] Previous researchers reported that PKC activators such as phorbol 12-myristate 13-acetate produce large increases in TACE activity which correlated with increasd sAPPα and decreased Aβ, suggesting that TACE and BACE1 compete for availability of APP substrate, and that PKC activators shift the competition in favor of TACE. Buxbaum et al., *J. Biol. Chem.* 1998; 273: 27765-67; Etcheberrigaray et al., *Proc. Natl. Acad. Sci.* USA. 2006: 103:8215-20. However, many of these earlier studies were carried out in fibroblasts and other non-neuronal cell types, which appear to respond differently to PKC activators than neurons. For example, Etcheberrigaray et al. found that activation of PKC in human fibroblasts by 10 pM to 100 pM bryostatin increased the initial rate of α-secretase activity by 16-fold and 132-fold, respectively (Etcheberrigaray et al., *Proc. Natl. Acad. Sci.* USA. 2006). However, in human SH-SY5Y neuroblastoma cells, N2a mouse neuroblastoma cells, and primary neurons from rat hippocampus, PKC activators bryostatin, **BR-101** (DCP-LA) and/or **BR-111** (DHA-CP6) only produced small increases in TACE activity. This suggests that any reduction of Aβ levels in neurons by PKC activators must be caused by some other mechanism besides activation of TACE.

Example 6: Effects of PKC Activators on Endothelin-Converting Enzyme Activity

[00200] ECE assay. SH-S757 neuroblastoma cells were incubated with bryostatin (0.27 nM), **BR-101** (DCP-LA) (1 μM), and **BR-111** (DHA-CP6) (1 μM). Endothelin-converting enzyme (ECE) was measured fluorimetrically using the method of Johnson and Ahn, *Anal. Biochem.* 2000; 286: 112-118. A sample of cell homogenate (20 μl) was incubated in 50 mM MES-KOH, pH 6.0, 0.01% C12E10 (polyoxyethylene-10-lauryl ether), and 15 μM McaBK2 (7-Methoxycoumarin-4-acetyl [Ala7-(2,4-Dinitrophenyl)Lys9]-bradykinin trifluoroacetate salt) (Sigma-Aldrich). After 60 min at 37 °C , the reaction was quenched by adding trifluoroacetic acid to 0.5%. The sample was diluted to 1.4 ml with water and the fluorescence was measured at ex = 334 nm, em = 398 nm.

Results and Discussion

[00201] Aβ can be degraded *in vivo* by a number of enzymes, including insulin degrading enzyme (insulysin), neprilysin, and ECE. Because PKCε overexpression has been reported to activate ECE (Choi et al., *Proc. Natl. Acad. Sci.* USA. 2006; 103: 8215-20), we examined the effect of PKC activators on ECE. Bryostatin, **BR-101** (DCP-LA), and **BR-111** (DHA-CP6) all produced a sustained increase in ECE activity. Since ECE does not possess a diacylglycerol-binding C1 domain, this suggests that the activation by bryostatin was not due to direct activation of ECE, but must have resulted from phosphorylation of ECE or some ECE-activating intermediate by PKC. This result also suggests that indirect activation ECE by PKC activators could be a useful means of reducing the levels of Aβ in patients.

[00202] An advantage of compounds such as the PUFA derivatives of the present invention which specifically activate PKCε is that they produce less down-

regulation than phorbol esters and similar 1,2-diacylglycerol (DAG) analogues. The biphasic response of PKC to DAG-based activators means that a PKC activator may reduce Aβ levels at one time point and increase them at another. da Cruz e Silva et al., *J. Neurochem.* 2009; 108: 319-330. Careful dosing and monitoring of patients would be required to avoid effects opposite to those that are intended. Because of the relative inability of this new class of PKC activators to downregulate PKC, this problem can be avoided.

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[00203] All patents, references and printed publications cited in the instant specification are hereby incorporporated by reference herein in their entireties.

WHAT IS CLAIMED IS:

1) A method of diagnosing Alzheimer's Disease in a human subject, said method comprising the steps of:

a) determining the PKC epsilon level in said human subject; and

b) comparing the PKC epsilon level in said human subject to the PKC epsilon level in a control subject;

wherein said method is indicative of Alzheimer's Disease in said human subject if the PKC epsilon level in said human subject is lower than the PKC epsilon level in said control subject.

2) The method of claim 1, wherein said PKC epsilon level is measured in one or more cells.

3) The method of claim 1, wherein said PKC epsilon level is a PKC epsilon protein level or a PKC epsilon activity level.

4) The method of claim 1, wherein said PKC epsilon level is measured by RT-PCR.

5) The method of claim 1, wherein said control subject does not have Alzheimer's Disease.

6) The method of claim 1, wherein said determining step (a) is done *in vitro*.

7) The method of claim 2, wherein said cell is a fibroblast, buccal mucosal, neuron, or blood cell.

8) The method of claim 1 wherein said determining step (a) comprises a method selected from the group consisting of radioimmunoassay, Western blot assay, immunofluorescent assay, enzyme immunoassay, immuno-precipitation, chemiluminescent assay, immunohistochemical assay, dot blot assay and slot blot assay.

9) The method of claim 1, wherein the absence of Alzheimer's Disease in said human subject is indicated if said PKC epsilon level in said human subject is greater than or equal to the PKC epsilon level in said control subject.

10) A method of diagnosing Alzheimer's Disease in a human subject comprising the steps of:

a) obtaining one or more cells from a human subject;

b) determining the PKC epsilon level in said one or more cells;

c) contacting said one or more cells of step (a) with an agent that is a PKC epsilon activator;

d) determining the PKC epsilon level in said one or more cells in step (c) after said contacting in step (c);

wherein Alzheimer's Disease is indicated in said human subject if the PKC epsilon level determined in step (d) is greater that the PKC epsilon level determined in step (b).

11) The method of claim 10, wherein said PKC epsilon level is a PKC epsilon protein level or a PKC epsilon activity level.

12) The method of claim 10, wherein said PKC epsilon level is measured by RT-PCR.

13) The method of claim 10, wherein said PKC epsilon level is measured in one or more cells.

14) The method of claim 10, wherein said steps (b), (c) and (d) are done *in vitro*.

15) The method of claim 10, wherein said cell is a fibroblast, buccal mucosal, neuron, or blood cell.

16) The method of claim 10 wherein said determining steps (b), (d) or both (b) and (d) comprises a method selected from the group consisting of radioimmunoassay, Western blot assay, immunofluorescent assay, enzyme immunoassay, immuno-precipitation, chemiluminescent assay, immunohistochemical assay, dot blot assay and slot blot assay.

17) The method of claim 10, wherein the absence of Alzheimer's Disease in said human subject is indicated if said PKC epsilon level in determined in step (d) is equal to or less than the PKC epsilon level determined in step (b).

18) A method of determining or monitoring Alzheimer's Disease progression in a human subject comprising the steps of:

a) determining the PKC epsilon level in said human subject;

b) comparing the PKC epsilon level in said human subject to the PKC epsilon level in a control subject; and

c) determining or monitoring said Alzheimer's Disease progression based on said comparison in step (b).

19) The method of claim 18, wherein the PKC epsilon level in said human subject decreases as Alzheimer's Disease progresses.

20) The method of claim 18, wherein said PKC epsilon level is measured in one or more cells.

21) The method of claim 18, wherein said PKC epsilon level is a PKC epsilon protein level or a PKC epsilon activity level.

22) The method of claim 18, wherein said PKC epsilon level is measured by RT-PCR.

23) The method of claim 18, wherein said control subject does not have Alzheimer's Disease.

24) The method of claim 1, wherein said determining step (a) is done *in vitro*.

25) The method of claim 20, wherein said cell is a fibroblast, buccal mucosal, neuron, or blood cell.

26) The method of claim 18 wherein said determining step (a) comprises a method selected from the group consisting of radioimmunoassay, Western blot assay, immunofluorescent assay, enzyme immunoassay, immuno-precipitation, chemiluminescent assay, immunohistochemical assay, dot blot assay and slot blot assay.

27) The method of claim 18, wherein the PKC epsilon level increases in said human subject as Alzheimer's Disease progression is reversed.

28) A method for elevating the PKC epsilon protein level in a cell, comprising the step of contacting one or more human cells with an amount of a PKC activator effective to elevate the PKC epsilon protein level in said cell compared to an uncontacted human cell.

29) The method of claim 28, wherein said human cell is a fibroblast, buccal mucosal, neuron, or blood cell.

30) The method of claim 28, wherein said PKC activator is a macrocyclic lactone.

31) The method of claim 30, wherein said macrocyclic lactone is a bryostatin.

32) The method of claim 31, wherein said bryostatin is bryostatin-1.

33) The method of claim 28, wherein said PKC epsilon level is a PKC epsilon protein level or a PKC epsilon activity level.

34) A method of diagnosing Alzheimer's Disease in a human subject comprising the steps of:

a) obtaining one or more cells from a human subject;

b) determining the PKC epsilon level in said one or more cells;

c) contacting said one or more cells of step (a) with an Aβ peptide;

d) determining the PKC epsilon level in said one or more cells in step (c) after said contacting in step (c);

wherein Alzheimer's Disease is indicated in said human subject if the PKC epsilon level determined in step (d) is not significantly different from the PKC epsilon level determined in step (b).

35) The method of claim 34, wherein said PKC epsilon level is a PKC epsilon protein level or a PKC epsilon activity level.

36) The method of claim 34, wherein said Aβ peptide is $A\beta_{1-42}$.

37) The method of claim 34, wherein said PKC epsilon level is measured by RT-PCR.

38) The method of claim 34, wherein said PKC epsilon level is measured in one or more cells.

39) The method of claim , wherein said steps (b), (c) and (d) are done *in vitro*.

40) The method of claim 34, wherein said cell is a fibroblast, buccal mucosal, neuron, or blood cell.

41) The method of claim 34 wherein said determining steps (b), (d) or both (b) and (d) comprises a method selected from the group consisting of radioimmunoassay, Western blot assay, immunofluorescent assay, enzyme immunoassay, immuno-precipitation, chemiluminescent assay, immunohistochemical assay, dot blot assay and slot blot assay.

42) The method of claim 34, wherein the absence of Alzheimer's Disease in said human subject is indicated if said PKC epsilon level in determined in step (d) is less than the PKC epsilon level determined in step (b).

43) A kit comprising one or more antibodies specific for PKC epsilon.

44) The kit of claim 43, wherein said kit further comprises a PKC activator.

45) The kit of claim 43, wherein said kit further comprises a PKC epsilon activator.

46) The kit of claim 43, wherein said kit further comprises one or more oligonucleotides specific for a gene encoding PKC epsilon.

47) A kit comprising one or more oligonucleotides specific for a gene encoding PKC epsilon.

48) The kit of claim 47, wherein said kit further comprises a PKC activator.

49) The kit of claim47, wherein said kit further comprises a PKC epsilon activator.

50) A method of identifying a compound useful for the treatment of Alzheimer's Disease comprising:

a) obtaining one or more cells from an Alzheimer's Disease subject;

b) determining the PKC epsilon level in said one or more cells;

c) contacting said cells with a candidate compound;

d) determining the PKC epsilon level in said one or more cells after said contacting step (c);

wherein said candidate compound is identified as a compound useful for the treatment of Alzheimer's Disease if the PKC epsilon level determined in step (d) is greater than the PKC epsilon level determined in step (b).

51) The method of claim 50, wherein said PKC epsilon level is a PKC epsilon protein level or a PKC epsilon activity level.

52) The method of claim 50, wherein said PKC epsilon level is measured by RT-PCR.

53) The method of claim 50, wherein said steps are done *in vitro*.

54) The method of claim 50, wherein said cell is a fibroblast, buccal mucosal, neuron, or blood cell.

55) The method of claim 50 wherein said determining steps (b) or (d) or both comprises a method selected from the group consisting of radioimmunoassay, Western blot assay, immunofluorescent assay, enzyme immunoassay, immuno-precipitation, chemiluminescent assay, immunohistochemical assay, dot blot assay and slot blot assay.

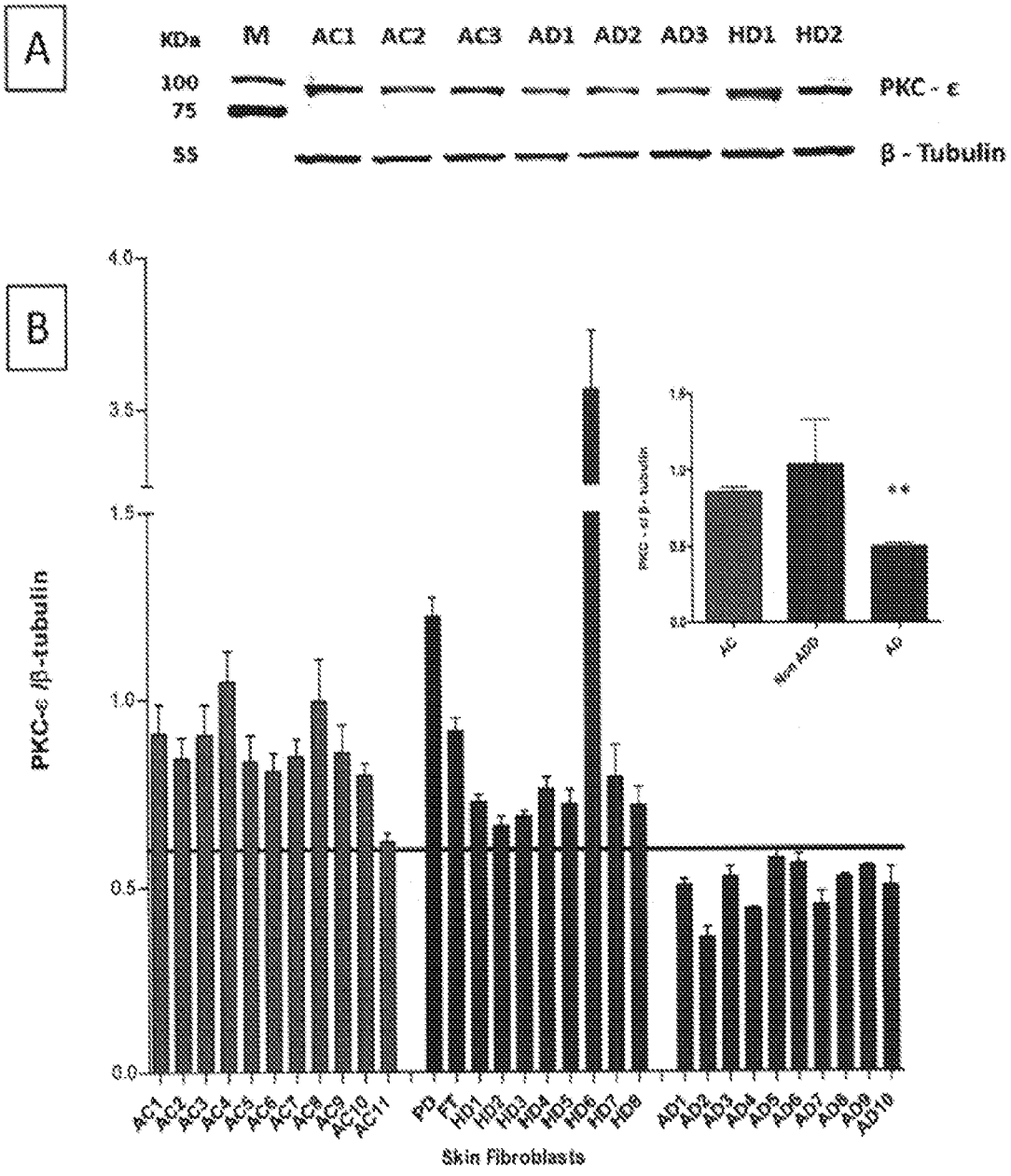

A
KDa
M
AC1
AC2
AC3
AD1
AD2
AD3
HD1
HD2
100
75
55
PKC - ε
β - Tubulin
B
PKC-ε /β-tubulin
4.0
3.5
1.5
1.0
0.5
0.0
**
AC
Skin HDF
AD
AC1 AC2 AC3 AC4 AC5 AC6 AC7 AC8 AC9 AC10 AC11
PD
FF
HD1 HD2 HD3 HD4 HD5 HD6 HD7 HD8
AD1 AD2 AD3 AD4 AD5 AD6 AD7 AD8 AD9 AD10
Skin Fibroblasts

Figure 1

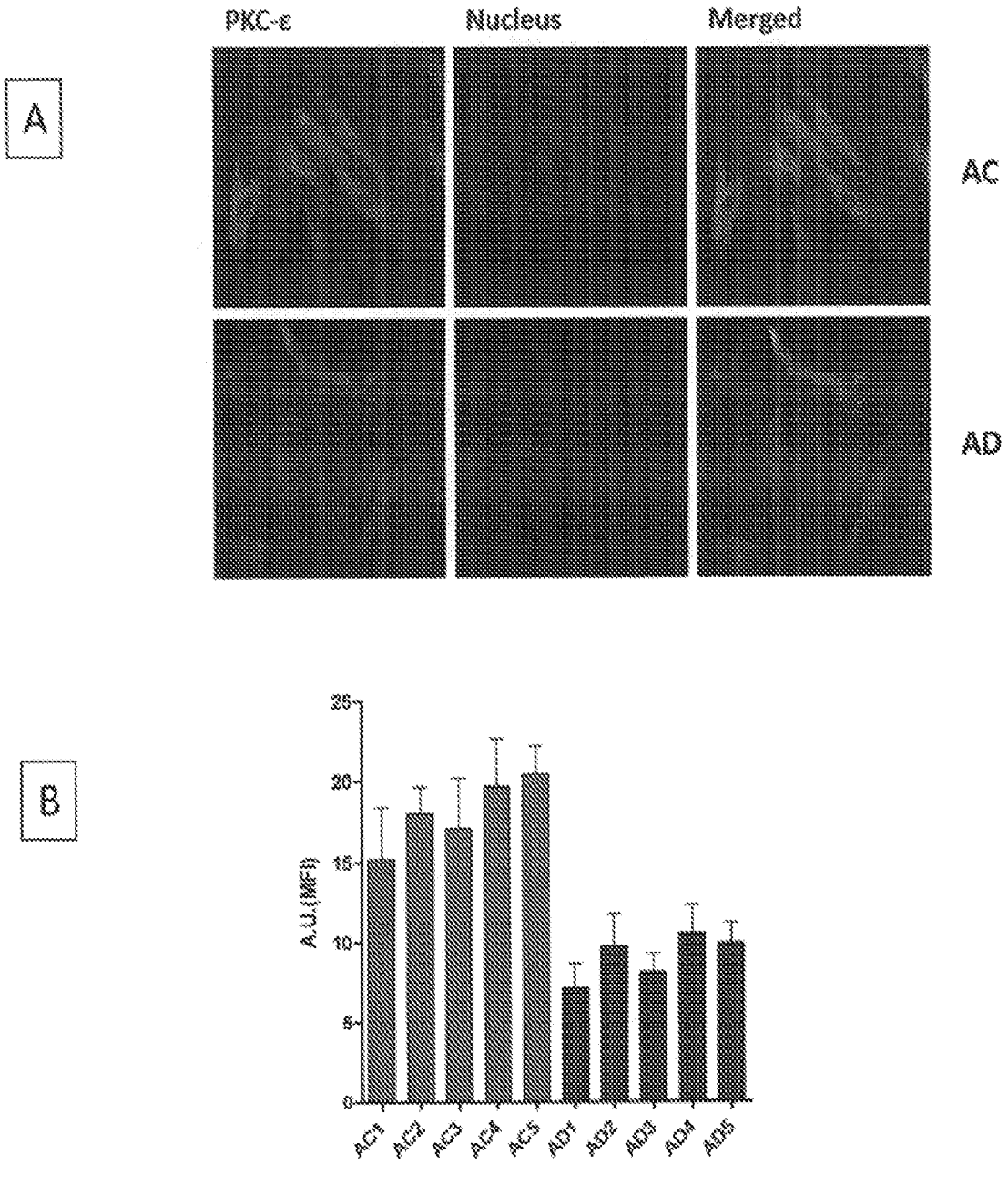

A
PKC-ε
Nucleus
Merged
AC
AD
B
A.U.(MFI)
0
5
10
15
20
25
AC1
AC2
AC3
AC4
AC5
AD1
AD2
AD3
AD4
AD5

Figure 2

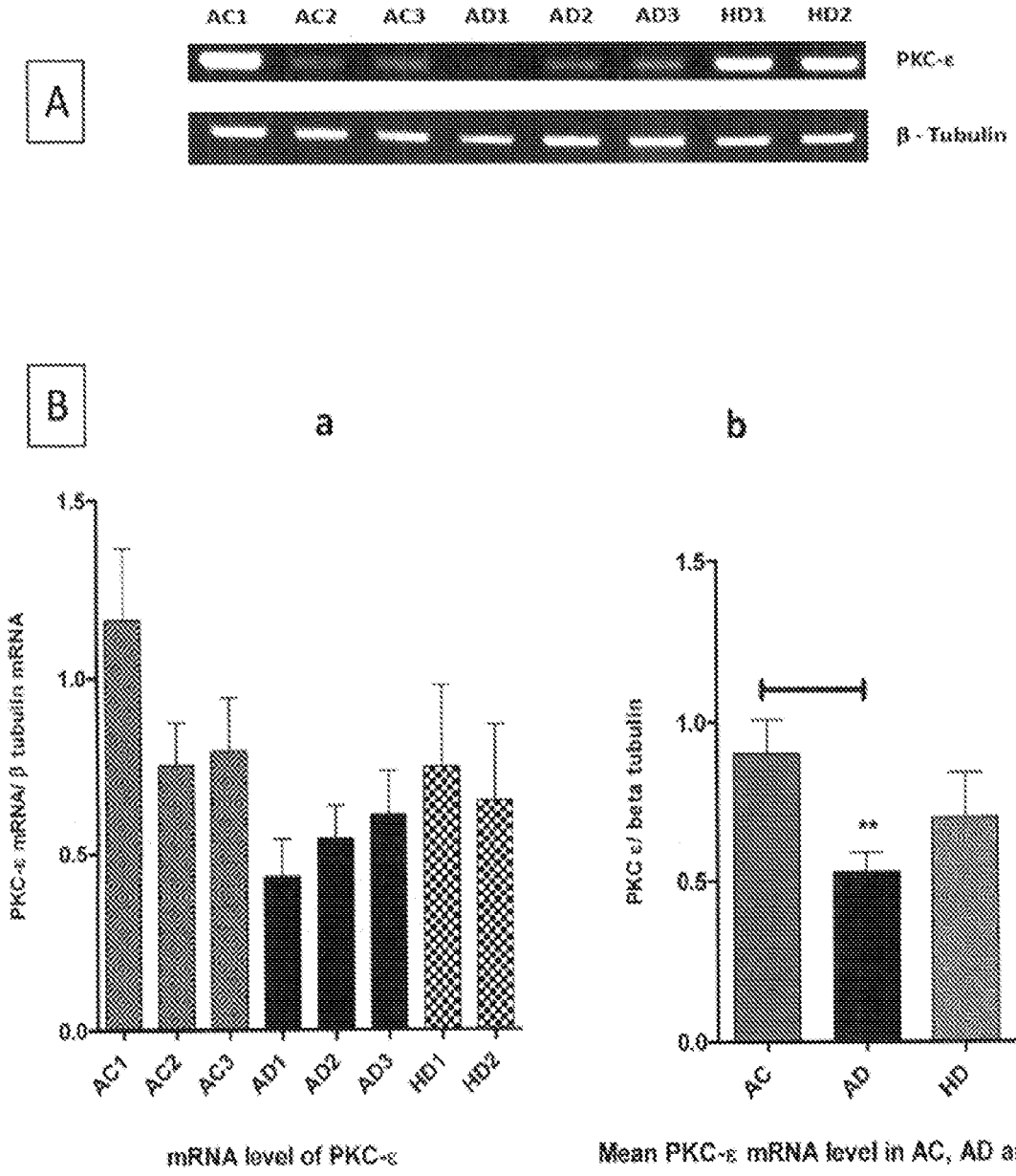

A
AC1
AC2
AC3
AD1
AD2
AD3
HD1
HD2
PKC-ε
β - Tubulin
B
a
b
PKC-ε mRNA/ β tubulin mRNA
1.5
1.0
0.5
0.0
AC1
AC2
AC3
AD1
AD2
AD3
HD1
HD2
mRNA level of PKC-ε
PKC ε/ beta tubulin
**
AC
AD
HD
Mean PKC-ε mRNA level in AC, AD and HD

Figure 3

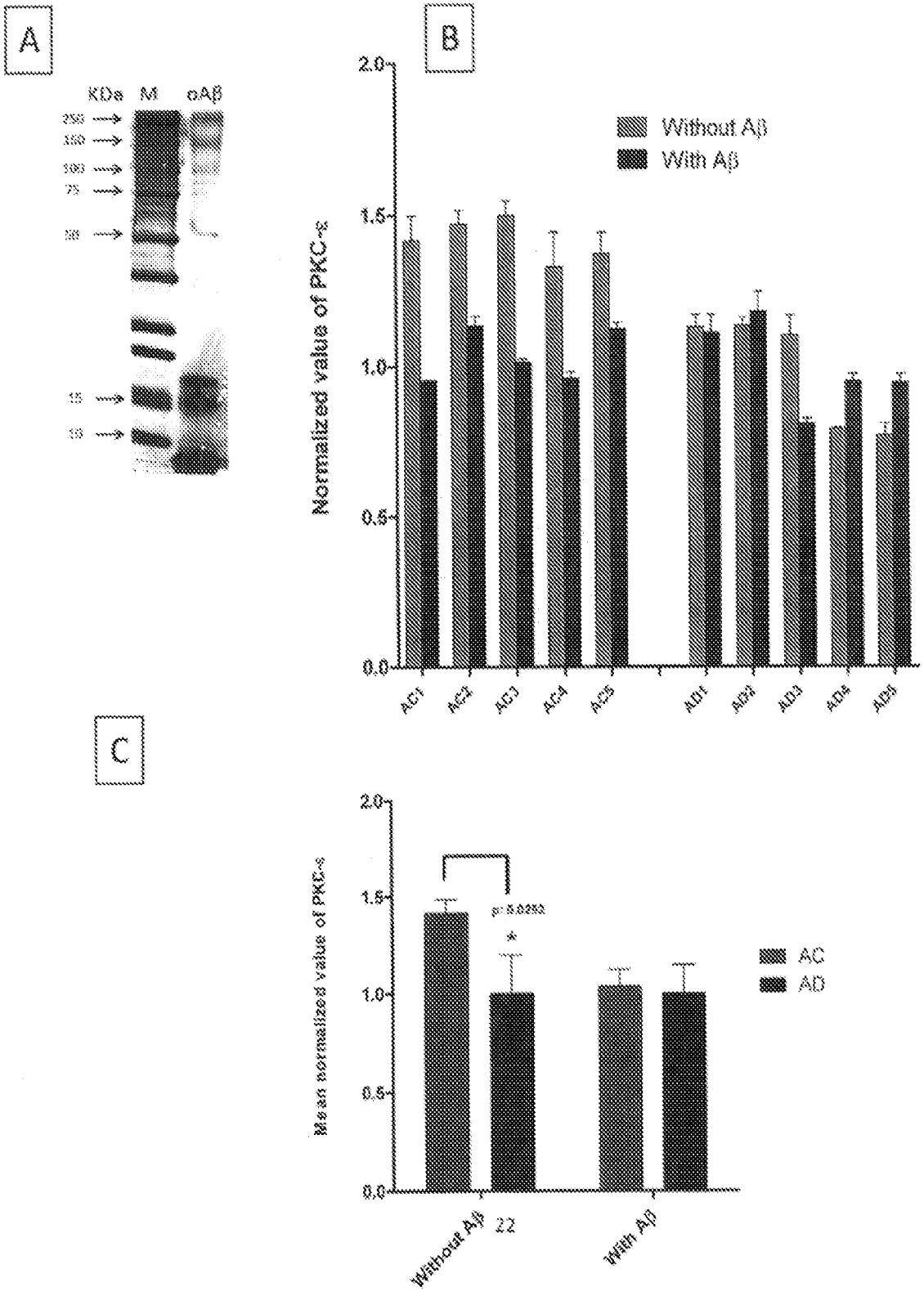

A
KDa
M
oAβ
250
150
100
75
50
15
10
B
Without Aβ
With Aβ
Normalized value of PKC-ε
2.0
1.5
1.0
0.5
0.0
AC1
AC2
AC3
AC4
AC5
AD1
AD2
AD3
AD4
AD5
C
Mean normalized value of PKC-ε
p= 0.0262
*
AC
AD
Without Aβ
With Aβ
22

Figure 4

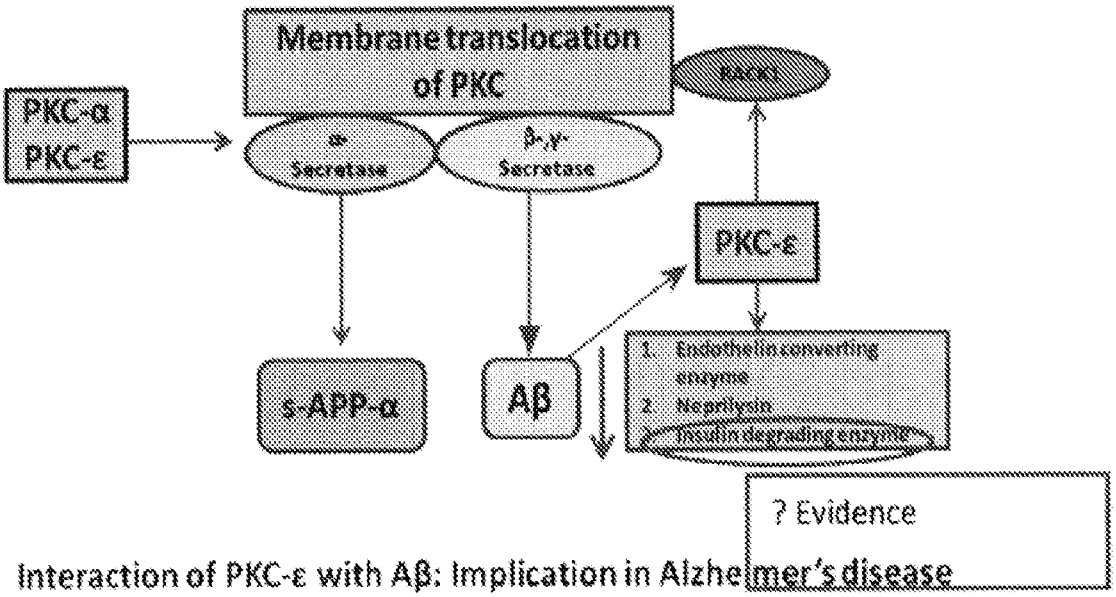

Membrane translocation of PKC
RACK1
PKC-α
PKC-ε
α-Secretase
β-,γ-Secretase
PKC-ε
s-APP-α
Aβ
1. Endothelin converting enzyme
2. Neprilysin
Insulin degrading enzyme
? Evidence
Interaction of PKC-ε with Aβ: Implication in Alzheimer's disease

Figure 5

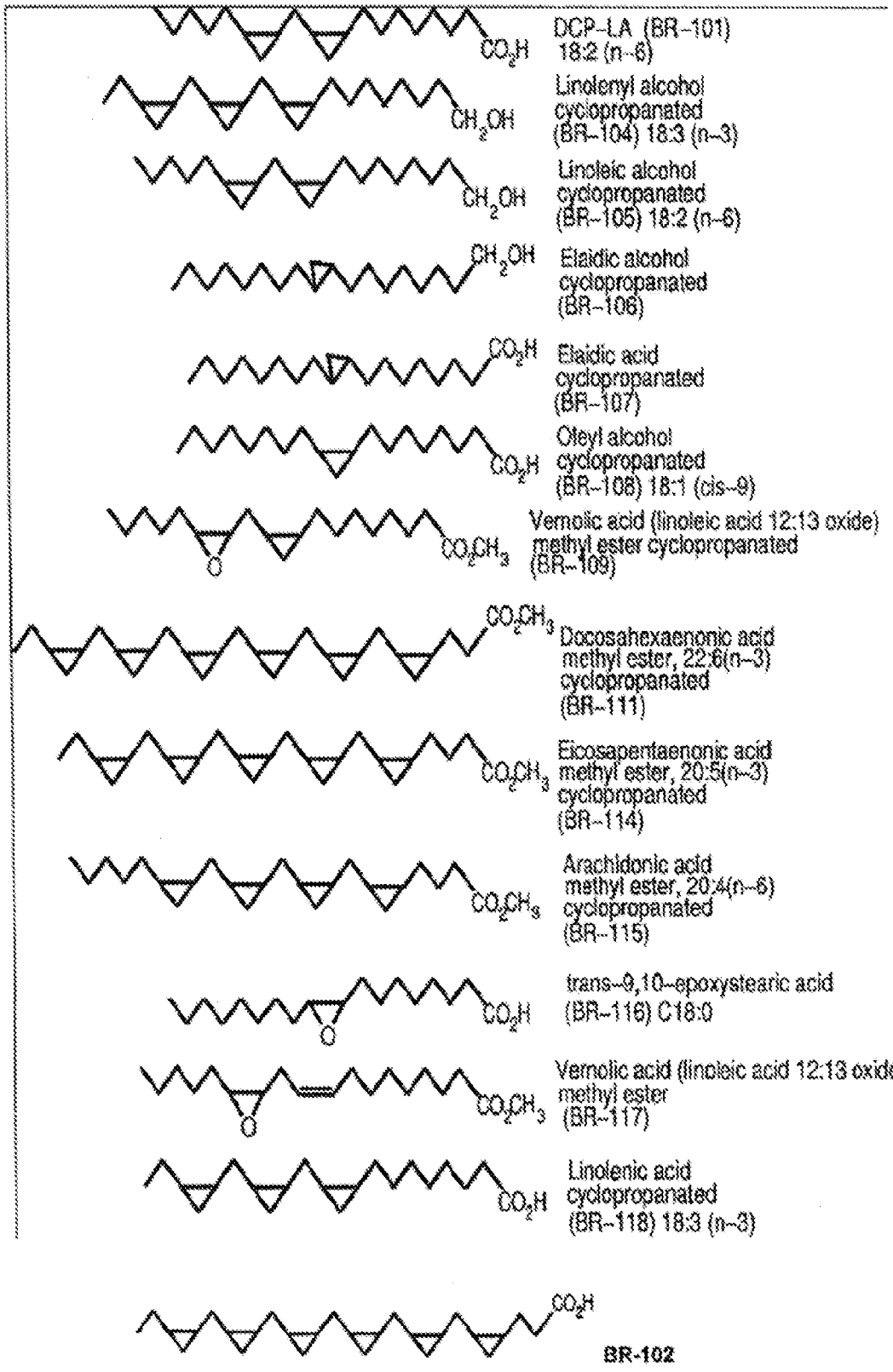

DCP-LA (BR-101)
18:2 (n-6)
Linolenyl alcohol
cyclopropanated
(BR-104) 18:3 (n-3)
Linoleic alcohol
cyclopropanated
(BR-105) 18:2 (n-6)
Elaidic alcohol
cyclopropanated
(BR-106)
Elaidic acid
cyclopropanated
(BR-107)
Oleyl alcohol
cyclopropanated
(BR-108) 18:1 (cis-9)
Vernolic acid (linoleic acid 12:13 oxide)
methyl ester cyclopropanated
(BR-109)
Docosahexaenonic acid
methyl ester, 22:6(n-3)
cyclopropanated
(BR-111)
Eicosapentaenonic acid
methyl ester, 20:5(n-3)
cyclopropanated
(BR-114)
Arachidonic acid
methyl ester, 20:4(n-6)
cyclopropanated
(BR-115)
trans-9,10-epoxystearic acid
(BR-116) C18:0
Vernolic acid (linoleic acid 12:13 oxide)
methyl ester
(BR-117)
Linolenic acid
cyclopropanated
(BR-118) 18:3 (n-3)
BR-102

Figure 6

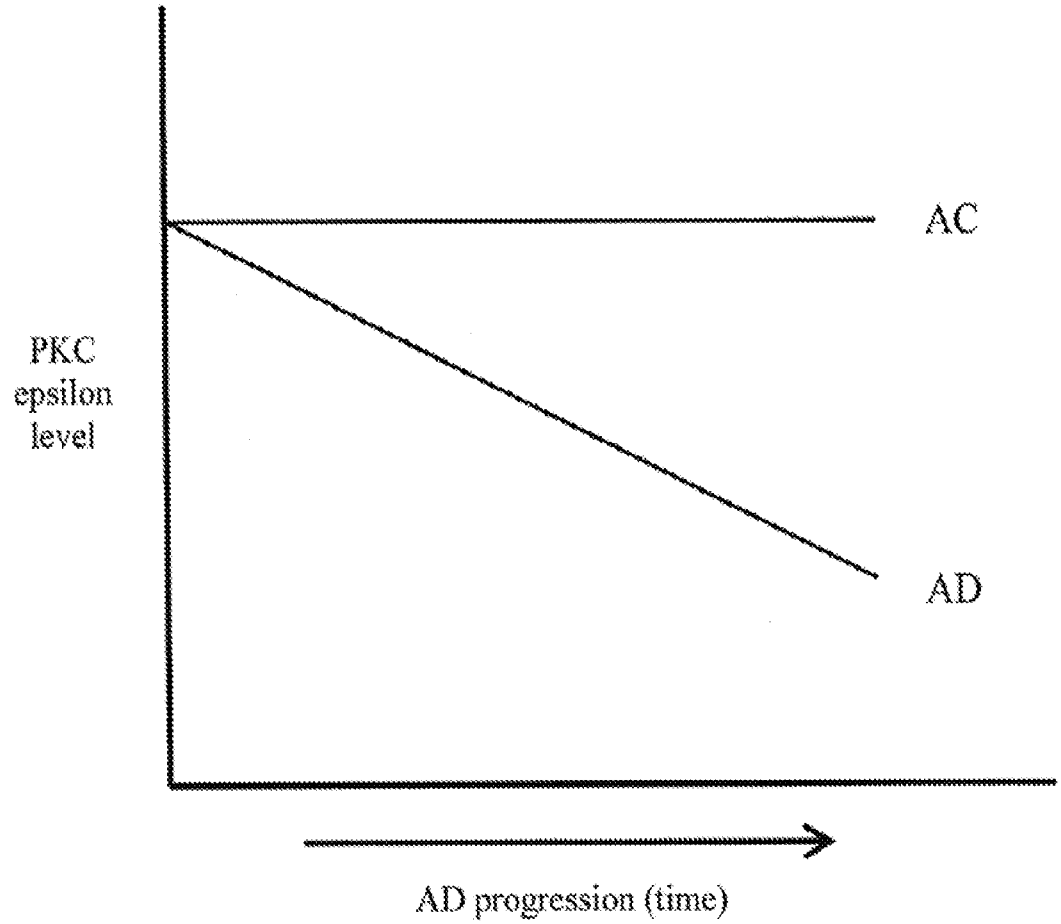


Figure 7

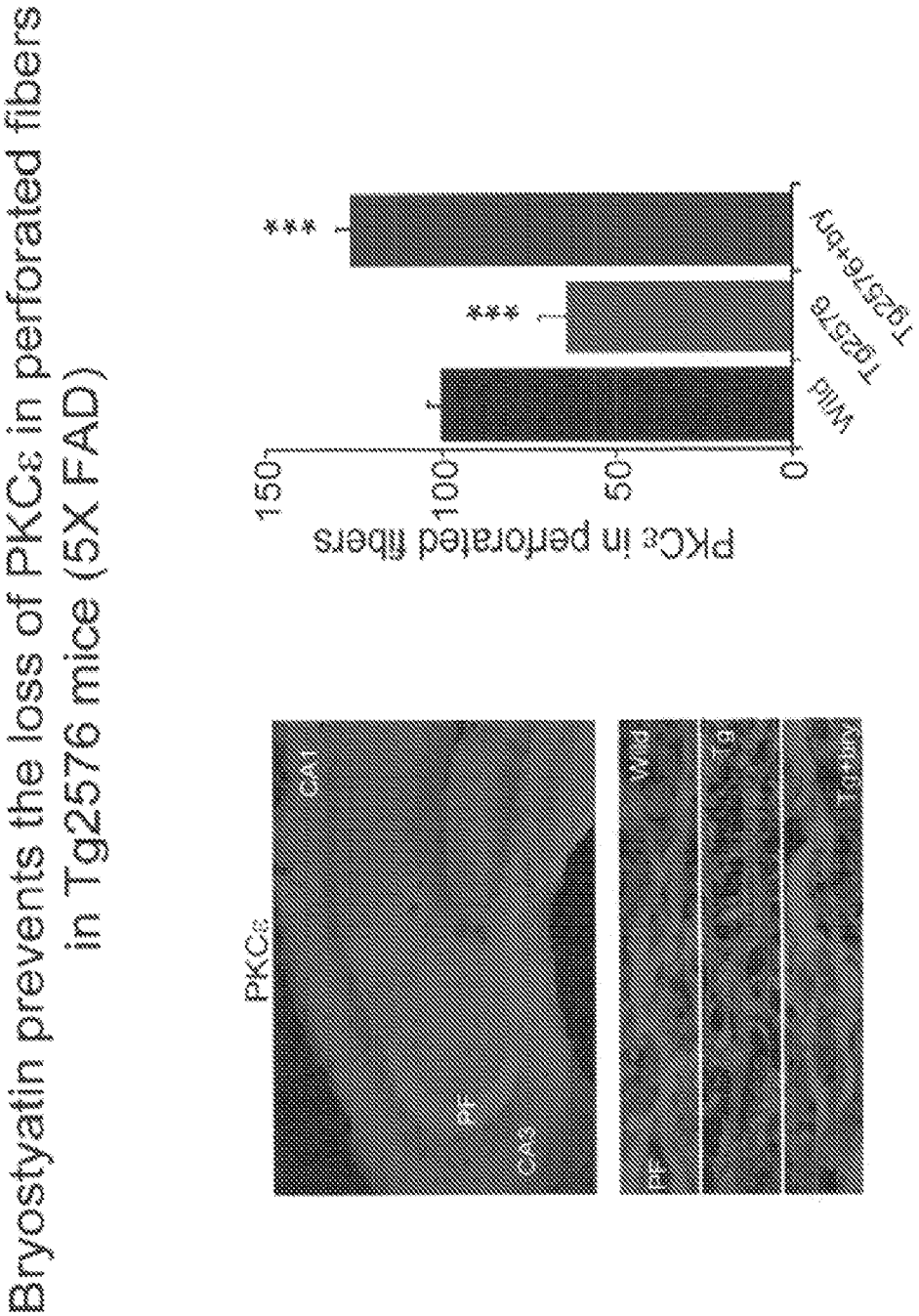
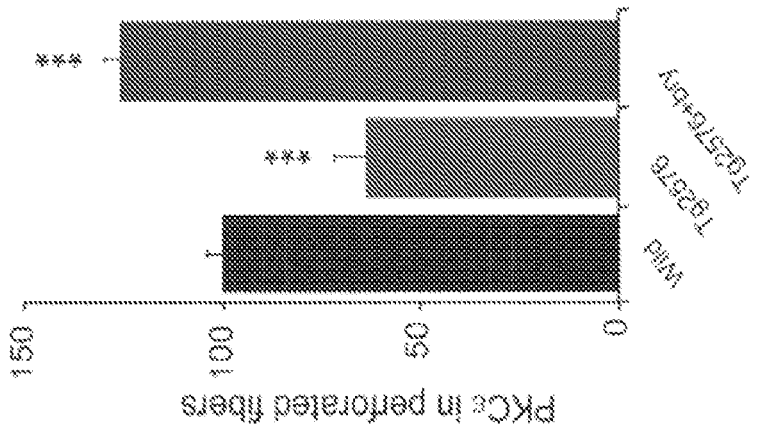

Bryostyatin prevents the loss of PKCε in perforated fibers
in Tg2576 mice (5X FAD)
PKCε in perforated fibers
150
100
50
0
***
***
Wild
Tg2576
Tg2576+bry
PKCε
CA1
SP
CA3
DG
ML
IML

Figure 8


PKCε in perforated fibers
0
50
100
150
wild
T235Tb
T235Tb+Ionomycin
***
***

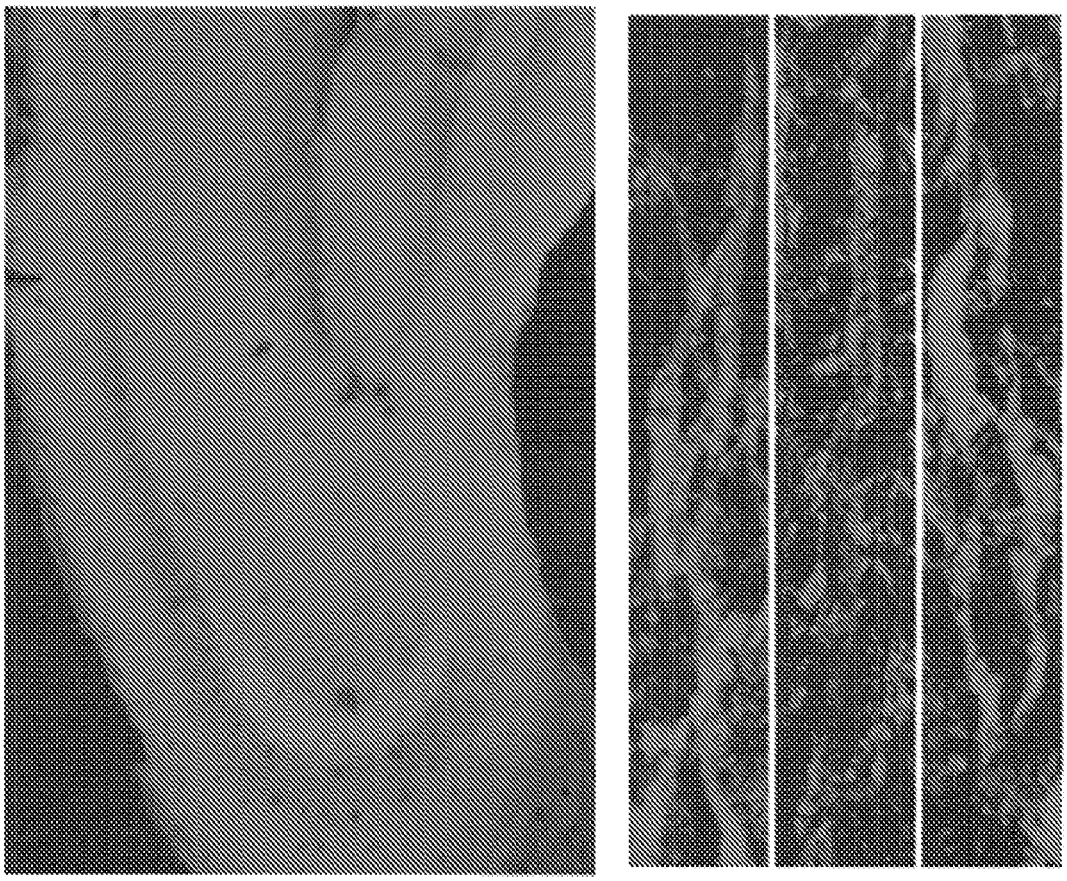


Figure 9

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US 11/00315

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - G01N 33/48, 33/53 (2011.01)
USPC - 435/6, 435/7.1; 436/86
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)
IPC(8) - G01N 33/48, 33/53 (2011.01)
USPC - 435/6, 435/7.1; 436/86; 435/4; 436/501

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
IPC(8) - G01N 33/48, 33/53 (2011.01) - see keyword below
USPC - 435/6, 435/7.1; 436/86; 435/4; 436/501 - see keyword below

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST(USPT,PGPB,EPAB,JPAB); Medline, Google: diagnosing Alzheimer's, PKC epsilon, PKCepsilon, protein kinase C, Protein kinase C-epsilon, PKC-epsilon, activator, protein level, activity, RT-PCR, fibroblast, buccal mucosal, neuron, blood cell, amyloid beta, Abeta, fibroblast, buccal mucosal, neuron, blood, radioimmunoassay, Western, immunofluor

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 6,107,050 A (ALKON et al.) 22 August 2000 (22.08.2000), Abstract; col 2, ln 61-62; col 4, ln 6-13 and 29-30; col 9, ln 37-38 and 50-52; col 15, ln 54-58; col 16, ln 11-13; col 19, ln 15-21; col 20, ln 1-4; and col 44, ln 59-65	1-27, 34-42
Y	US 2010/0022645 A1 (NELSON et al.) 28 January 2010 (28.01.2010), para [0003], [0010], [0014], [0030], [0054], [0110], [0115], [0119], [0127], and [0132]	1-27, 34-42
Y	SAKAI et al. Identification of PKC isoforms expressed in human bronchial smooth muscle cell. J. Smooth Muscle Res. 2009, Vol. 45 (1), p. 55?62. Abstract; and pg 58, Talbe 1	4, 12, 22, 37
A	LEE et al. Amyloid beta peptide directly inhibits PKC activation. Mol Cell Neurosci. 2004, Vol. 26(2), p. 222-31. Abstract	1-27, 34-42
A	ZHU et al. Protein kinase C epsilon suppresses Abeta production and promotes activation of alpha-secretase. Biochem Biophys Res Commun. 2001, Vol. 285(4), 997-1006. Abstract	1-27, 34-42
A	US 2010/0021913 A1 (ALKON et al.) 28 January 2010 (28.01.2010), Abstract	1-27, 34-42
A	US 2009/0130195 A1 (ACEVEDO-DUNCAN et al.) 21 May 2009 (21.05.2009), Abstract, para [0033]	1-27, 34-42
A	DEACON et al. Isoenzymes of protein kinase C: differential involvement in apoptosis and pathogenesis. Mol Pathol. 1997, Vol. 50(3), 124-31. pg 148, col 1, para 4	1-27, 34-42

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"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
15 July 2011 (15.07.2011)	02 AUG 2011
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

PCT/US 11/00315

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: claims 1-27 and 34-42, directed to a method of diagnosing or monitoring Alzheimer's disease in a human subject, comprising the steps of: a) determining the PCK epsilon level in a subject; and b) comparing the PKC epsilon level to the PKC epsilon level in a control subject, wherein the level of PKC epsilon is lower in the test subject than in the control.

Group II: claims 28-33, directed to a method for elevating PKC epsilon protein levels in a cell, comprising contacting one or more human cells with a PKC activator.

- Please see extra sheet for continuation -

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-27 and 34-42

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Continuation of Box III: Lack of Unity of Invention

Group III: claims 43-46, directed to a kit comprising one or more antibodies specific for PKC epsilon.
Group IV: claims 47-49, directed to a kit comprising one or more oligonucleotides specific for a gene encoding PKC epsilon.
Group V: claims 50-55, directed to a method of identifying a compound useful for treatment of Alzheimer's Disease, comprising: a) obtaining one or more cells from an Alzheimer's Disease subject; b) determining the PKC epsilon levels in said cells; c) contacting said cells with a candidate compound; and d) determining the PKC epsilon level in said cells after said contacting step, wherein a candidate compound is identified as useful for the treatment of Alzheimer's if the PKC level is increased by the compound.

The inventions listed as Groups I - V do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The special technical feature of the Group V claims is providing a method of screening candidate compounds useful for treatment of Alzheimer's Disease - not required by the claims of any other Group. The special technical feature of the Group IV claims is a kit comprising one or more oligonucleotides specific for a gene encoding PKC epsilon - not required by the claims of any other Group. The special technical feature of the Group III claims is a kit comprising one or more antibodies specific for PKC - not required by the claims of any other Group. The special technical feature of the Group II claims is a method for elevating PKC epsilon levels in a cell, comprising contacting one or more cells with a PKC activator - not required by the claims of any other Group. The special technical feature of the Group I claims is a method of diagnosis, or montioring Alzheimer's Disease in a subject - not required by the claims of any other Group.

The only common technical element shared by all of the above groups is that they are related to protein kinase c epsilon. The claims of Group I and Group V share a further common technical element of being related to assessing the level of expression or activity of PKC epsilon. This common technical element does not represent an improvement over the prior art of US 20090130195 A1 to Acevedo-Duncan et al., which discloses a method of detecting prostate tumorigenesis in a subject, including the steps of (a) obtaining a sample from the prostate of the human subject, (b) detecting quantitatively or semi-quantitatively in the sample a level of expression for PKC- and (c) comparing the expression level in (b) to a level of expression in a normal control (abstract), wherein the PKC detected may be PKC epsilon (PKC-.epsilon. is being targeted for prostate therapy; para [0033]). The claims of Groups I and V share the further common technical element of being related to PKC epsilon expression in Alzheimer's Disease. This common technical element does not improve upon the prior art of the article entitled "Isoenzymes of protein kinase C: differential involvement in apoptosis and pathogenesis" by Deacon et al., which discloses wherein PKC epsilon is decreased in Alzheimer's disease (pg. 128, col 1, para 4). A further common technical element is shared by Group II and Group V, wherein expression or activity of PKC epsilon may be increased in response to a stimulator. This common technical element does not improve upon the prior art of US 2010/0022645 A1 to Nelson et al. Discloses the activation of PKC epsilon (para [0110], [0115]) in association with the treatment of Alzheimer's (abstract). Therefore, the inventions of Groups I-V lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.

专利名称(译)	蛋白激酶cε（pkc-epsilon）蛋白水平的阿尔茨海默氏病特异性改变		
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当前申请(专利权)人(译)	布兰切特洛克菲勒神经科学		
[标]发明人	KHAN TAPAN K ALKON DANIEL L		
发明人	KHAN, TAPAN, K. ALKON, DANIEL, L.		
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摘要(译)

本发明涉及通过检测人患者中PKCε蛋白水平与对照受试者中PKCε水平相比的变化来诊断人患者的阿尔茨海默氏病的方法。本文公开的阿尔茨海默氏病特异性分子生物标志物可用于诊断阿尔茨海默氏病和用于鉴定用于治疗或预防阿尔茨海默氏病的化合物的筛选方法。本发明还提供了提高PKCε蛋白水平的方法，包括使一种或多种人细胞与一定量PKC活化剂接触的步骤，与未接触的人细胞相比，所述PKC活化剂有效提高PKCε水平。