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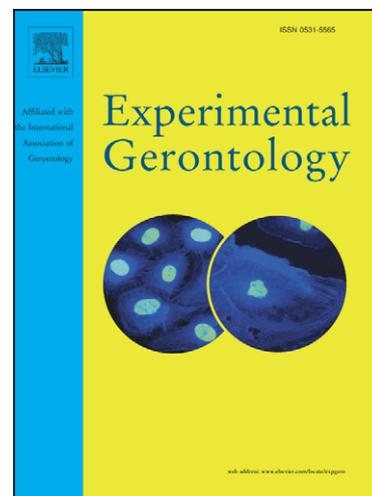
Protein kinase C as a peripheral biomarker for Alzheimer's disease

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Protein kinase C as a peripheral biomarker for Alzheimer's disease**Jean de Barry^{1*}, Corinne Mbebi Liégeois² and Agnes Janoshazi³**¹Institut des Neurosciences Cellulaires et Integratives, CNRS, Strasbourg, France ;²Innovative Health Diagnostics, Strasbourg, France ; ³Institut de Science et d'Ingenierie Supramoleculaires, Université de Strasbourg, France.

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Running title: PKC in AD blood cells**Key words: Alzheimer's disease, beta-amyloid, biomarker, erythrocyte, red blood cell, human, Parkinson's disease, PKC.**

Abstract

Numerous studies have been performed, which assess an important role of protein kinase C (PKC) in the pathophysiology of Alzheimer disease (AD). The alteration of PKC activity stimulates amyloid-beta peptides production and protein tau hyperphosphorylation. This recently led to consider PKC as a potential therapeutic target for disease modifying drugs. Moreover PKC alterations were also observed in peripheral cells including blood cells. This short review recalls the main findings on the role of PKC in the disease process and focuses on its use as an AD biomarker in blood cells. Using fluorescent probes specific for PKC, it is possible to detect the conformational changes of the enzyme in living cells. Such probes can be used to detect PKC alterations in red blood cells and thus to distinguish AD patients from healthy controls with unmatched specificity and sensitivity.

Introduction

A specific blood test would be the most desired diagnostic examination. It is expected to be minimally invasive, easy to perform in a general care setting, and cost effective. Since there are numerous compounds presently in a preclinical phase or in phase II clinical studies, the focus has shifted again to finding a screening test for cohort recruitment and stratification. On a long term, such a test could be performed in general clinical laboratories accessible to the elderly population even in remote areas. Additionally, provided that disease modifying drugs were available, this test should be useful for treatment monitoring.

Serum (plasma) or red blood cell investigations may, as well, be more promising for a population-screening test than any current practice. The downside of alterations observed in the periphery is that they may be less specific for CNS disorders, unless consequences of specific features of the disease are taken into account as additional parameters. Nevertheless, even markers that have little discriminative value on their own might be useful for the diagnosis in combination with other tests.

The search for a peripheral biochemical marker of the disease has resulted in the discovery of numerous changes that take place in the blood of Alzheimer's disease (AD) patients. Beyond changes in the composition of the plasma, changes also occur in the cellular constituents of blood and among other biomarkers, PKC has been found to be altered in blood cells (Janoshazi et al., 2006).

We will briefly focus on the role of PKC in the physiopathology of Alzheimer's disease and describe the alterations of PKC observed in blood cells from AD patients.

The PKC family and its role in memory processes

The PKC family includes at least 12 different isoforms classified in 3 different subclasses (conventional, novel and atypical), according to their sequence homology and their activation requirements. It is generally accepted that the PKC activation process implies a phosphorylation, a conformational change and a translocation of the inactive cytosolic enzyme towards a cellular membrane (Luo and Weinstein, 1993; Keranen et al., 1995). In the membranes, PKC interacts with diacylglycerol and eventually with phorbol ester, which is a potent activator of the enzyme (Quest and Bell, 1994).

Indeed, PKC has a well established function in memory processes in animal models (Sun et al., 2009) and has been found to play an important role in the altered signal transduction pathway in AD (ref). Alterations have been described as decreased levels, activity and translocation of PKC in AD brain and an involvement in the alteration of the transduction system (for review see Pascale et al., 2007).

PKC and beta-amyloid peptide interactions

The processing of Amyloid Precursor Protein (APP) into the α - or β -secretory pathways is under the control of protein phosphorylation, in particular by PKC, a mechanism that has been shown defective in sporadic AD fibroblasts.

The activation of the α -secretase-mediated cleavage of APP is either direct by activation of PKC isozymes α and ϵ , or indirect through PKC activation of ERK1/2, or both. PKC can work directly on α -secretase and molecules such as the ADAM (a Disintegrin metalloproteinase) member of the tumor necrosis factor- α converting enzymes family (TACE) (Lammich et al., 1999). The phosphorylation involved in α -secretase cleavage of APP at the plasma membrane (i.e. on the cell surface) is enhanced by PKC stimulation. Phorbol ester-induced α -secretase activation apparently involves translocation of PKC α from the cytosol to

the membrane compartment, and translocation of PKC ϵ from cytosol to Golgi-like structures (Lanni et al., 2004).

Additionally, the MAPKs ERK1/2 (which are also phosphorylated by PKC) have been shown to phosphorylate TACE within a specific molecular domain (Rapoport and Ferreira, 2000; Chong et al., 2006), thus enhancing α -secretase activity.

However statins (cholesterol-lowering drugs)-induced increases of sAPP release (through α -secretase) are not blocked by either inhibitors of PKC or ERK1/2 (Buxbaum et al., 2001). This finding suggests that α -secretase can also be activated by a mechanism independent of PKC and MAPKs.

On the other hand, β -amyloid peptides (A β) can inactivate PKC.: A β contains a putative PKC pseudosubstrate site (A β ₂₈₋₃₀), which is critical for A β -PKC direct interaction (Lee W., 2004). A β ₁₋₄₀ has been reported to degrade PKC α in normal human fibroblasts and PKC γ in AD patient fibroblasts (Favit et al., 1998). A β ₁₋₄₀, at high concentrations, also reduced PKC-mediated phosphorylation of several soluble brain proteins in a liposome system (Chauhan A., 1991). Addition of A β ₁₋₄₀ peptides to cultured B103 cells reduced the activated forms of PKC α and PKC ϵ . It also inhibited phorbol ester-induced membrane translocation of PKC α and PKC ϵ without altering their expression levels, indicating that activation of intracellular PKC is inhibited by treatment with A β peptides (Lanni et al., 2004).

In patients who already have elevated A β concentration, this A β inhibition of PKC may act as a positive feedback, causing greater reduction of α -secretase activity and thus further production of A β . But this phenomenon is probably related to specific PKC isoforms: it has been shown that PKC ϵ reduces A β levels *in vitro* and *in vivo* (Zhu et al., 2001). PKC ϵ acts on the stimulation of A β degradation in the brains of PKC ϵ transgenic mice that express amyloidogenic variants of human APP (Choi et al. 2006).

Is PKC sensitive to the deleterious effects of A β oligomers ?

Recently it has been shown A β oligomers might be the culprits, inhibiting hippocampal long-term potentiation and disrupting synaptic plasticity (Walsh and Selkoe, 2004). One study suggested that A β oligomers composed of 12 A β monomers are related to memory disturbances in Alzheimer's disease transgenic mice, although no data for sporadic disease were presented (Lesne et al., 2006). The core mechanism for amyloid neurotoxicity involves increases in intracellular calcium in target neurons. For example, it has been shown that exposure of neuronal cells to the amyloid peptide results in sustained elevation of intracellular Ca²⁺. The mechanism that generates this sustained Ca²⁺ elevation is not totally understood but it is proposed to be due to a calcium channel activity intrinsic to the amyloid peptide itself or to oligomers inserted into the cellular membrane (Shankar et al., 2007). Evidence of consequent oxidative stress has also been reported in amyloid peptide treated cells (Sultana et al., 2009). Both sustained intracellular Ca²⁺ increase and oxidative stress through lipid peroxides can lead to PKC inactivation, but PKC isozymes are differently sensitive to such events.

PKC and Tau interaction

Almost in parallel with the identification of A β in plaques, tangles were shown to be composed of abnormally hyperphosphorylated tau protein. Tau is the major microtubule-associated structural protein in neurons and it stabilizes microtubule (MT). The MT-binding ability of tau is post-translationally regulated primarily by serine/threonine-directed phosphorylation, which can effectively modulate the binding affinity of tau for MTs (Mazanetz and Fischer, 2007). Thus, relatively frequent cycles of tau–MT binding (promoted

by dephosphorylation of tau) and detachment of tau from the MT (promoted by phosphorylation of tau) are needed in order to maintain effective axonal transport. Tau phosphorylation is regulated by the balance between multiple kinases: glycogen synthase kinase 3 (GSK3), cyclin-dependent kinase 5 (CDK5), the microtubule-affinity-regulating kinase (MARK) and ERK1/2 (Mazanetz and Fischer, 2007). Hyperphosphorylated tau disassembles from microtubules and becomes prone to aggregation into insoluble fibrils (paired helical filaments; PHF) and larger aggregates in tangles.

Fibrillar $A\beta_{1-42}$ induces ERK1/2 activation, which in turn can lead to hyperphosphorylation of tau and subsequent neurodegeneration. Furthermore, the toxic oligomeric forms of $A\beta$ induce neuronal death through activation of the ERK1/2 pathway. ERK1/2 can cause tau phosphorylation, but the most important kinase that phosphorylates tau is GSK-3 β (Takashima, 2006). PKC can inhibit GSK-3 β directly, thus reducing tau phosphorylation and neurofibrillary tangles (Isagawa et al., 2000), and indirectly through its effects on $A\beta$. PKC indirectly inhibits GSK-3 β by reducing production of $A\beta_{1-42}$, which is an activator of GSK-3 β . A very suggestive result, namely that cultured hippocampal neurons from tau-knockout mice treated with fibrillar $A\beta$ were not susceptible to $A\beta$ -induced toxicity, makes the connections between $A\beta$ and neurofibrillary tangles. $A\beta$ -mediated toxicity and tau pathology have repeatedly been proposed by the following links (Alkon et al., 2007): i- the reduction in levels of $A\beta_{1-42}$ by PKC through an α -secretase-mediated increase of sAPP, ii-the inhibition by PKC of GSK-3 β and thus of tau phosphorylation, and iii- together with $A\beta$ -mediated activation of GSK-3 β .

To conclude a deficiency of PKC activation could account for the following symptoms:

- (i) Memory loss – the characteristic presenting symptom of AD, (ii) Increased levels of $A\beta$ (owing to decreases in the activity of PKC, MAPK and α -secretase) and resulting amyloid

plaques,(iii) Increased levels of phosphorylated tau (reduced PKC-mediated inhibition of GSK-3 β) and resulting neurofibrillary tangles and (iv) Inflammation.

Insert Figure 1

Finally, age, the single greatest risk factor for the sporadic form of AD has been associated with progressive compromise of PKC function (for review see Pascale et al., 2007). Aged animal models, for example, have shown age-specific changes of PKC isozyme distribution in the brain, impaired PKC translocation, reduced levels of the PKC anchoring protein, RACK1 (receptor for activated C kinase 1), alterations in ERK1/2, and reduced levels of the α -secretase-cleaved APP product, sAPP α , in the cerebrospinal fluid (for review see Pascale et al. 2007).

PKC is probably not the primarily impaired protein in AD, but the consequences of its alteration provide a convergent causal link for two main features of the disease: A β production and tau hyperphosphorylation. Indeed this makes PKC a target of choice for developing disease modifying drugs (for a review see Sun and Alkon, 2006).

The next question arising is whether PKC is a convenient peripheral biomarker for AD.

Biomarkers in the blood

The use of peripheral tissues to diagnose the disease is based on the hypothesis that Alzheimer's disease might be a systemic disease that affects several tissues in the body.

Indeed the alterations of transduction systems in AD have been reported in numerous publications, in peripheral tissues, in some cases mirroring findings in the brain of AD patients. Hence two factors should be considered: the role of the blood brain barrier in the disease and systemic disturbances induced by the disease.

The blood brain barrier separates the central nervous system (CNS) from the rest of the body allowing for control of the substances that enter the neuronal environment. A β and tau are exported from the brain to the blood (DeMattos et al., 2002) and the blood brain barrier is definitively altered in AD patients (for a review see Bell and Zlokovic, 2009). However the A β peptide is not only produced in brain but has also been found in cerebrospinal fluid and blood plasma of normal individuals (Poduslo et al., 1999, 2001; Strazielle et al., 2000). Hence it is all too often overlooked that there is some A β generated extracerebrally so that not all the peripheral A β originates from the brain, a fact that calls for a cautious interpretation of any measurement of its peripheral levels.

PKC in blood cells

Changes were reported in brain PKC activity of AD patients (Pascale et al. 2007) and this observation was extended to peripheral tissues since PKC seems to be altered in fibroblasts (Govoni et al., 1993) and lymphocytes from AD patients (Solerte et al. 1998). Moreover, Band 3 (AE1), an anion exchanger transmembrane protein, which is important for organizing the structure of the red blood cells (RBC) membrane, which performs the same functions in brain as it does in erythrocytes (RBC) and which is phosphorylated by PKC, showed similar alteration in brain and RBC of AD patients. These parallel changes included decreased 32P-phosphate labeling, altered conformation of band 3 recognized by polyclonal and monoclonal antibodies and decreased anion transport in RBC (Kay and Goodman, 1997). This finding could suggest that one of the sources of this alteration of a major transport complex in two different cell types is either a general modification in PKC activity or changes in the membrane bilayer, which is stabilized by Band 3 in RCB. Further investigation proved that the PKC hypothesis was right.

Insert Figure 2

Conformational changes of PKC and its translocation towards the plasma membrane can be studied with simple fluorescent methods in living cells (Chen and Poenie, 1993; Dupont et al., 2000; Geeraert et al., 2003). We used Fim-1, a fluorescein-coupled bis-indoylmaleimide specific for PKC (Chen and Poenie, 1993). This compound binds to the ATP site in the catalytic domain of the enzyme and detects PKC conformational changes *in vitro* (Figure 2) and in intact cells (Dupont et al., 2000; Janoshazi and de Barry, 1999). After loading RBC with the fluorescent probe, fluorescence spectra were recorded in different conditions (with the probe alone or in the presence of staurosporine, an inhibitor of PKC, or PMA, a phorbol ester which activates PKC). A subsequent analysis of the spectra by deconvolution revealed spectral changes according to the measurement conditions. These changes were quantified by spectral deformation indices (SDI).

This protocol revealed a significant alteration of PKC conformation in intact RBC from AD patients (Janoshazi et al., 2006). Two groups were recruited : 35 healthy volunteers without psychiatric or neurologic past history and 35 aged-matched AD patients. AD patients fulfilled the NINCDS-ADRDA criteria for probable AD (McKhann et al., 1984). They were fully investigated with a standard neurological examination, an extensive neuropsychological assessment, routine blood investigations and neuroimaging. The selected patients displayed significant cortical atrophy, at least involving the mesiotemporal regions. Patients with a stroke were rejected, so that patients with vascular dementia or so-called mixed dementia could be reasonably ruled out. Other confounding causes of dementia, such as dementia with Lewy bodies, could also be ruled out, since our patients had no extrapyramidal sign on neurological examination, no history of hallucination, fall or fluctuation. Their cognitive pattern was typical of AD and the follow-up of the cohort confirmed the clinical diagnosis made at the first assessment.

Insert Figure 3

Among 7 calculated spectral distortion indices, three allowed to clearly distinguish AD patients from healthy volunteers. The sensitivity and the specificity of these indices were $> 95\%$. An example of discrimination is given in figure 3. Indeed the discriminative power of our measurement is based on a clinical diagnosis, whose specificity is around 90% .

We did not find differences between the different stages of the illness (mild, moderate, and severe) in our protocol and it is surprising that the distributions of SDI values were narrower for the AD patient population than for the controls, despite considerable variation in stage of disease (Figure 3). This indicates that AD patients form a more homogeneous population from this point of view than the controls and that the values of SDI are not dependent on the stage of the disease. This in turn suggests the presence of a switch mechanism, leading to alterations of the environment or the conformation of PKC shown by SDIs. This switch already occurs at mild stages of the disease or possibly even before a clinical diagnosis can be made. A probable early switch is of particular interest since there is several years delay on average between the first complaints by patients about memory losses and the clinical diagnosis of AD.

Insert Figure 4

The question was raised of whether a bias in the measurement was introduced by an age dependency of our indices. We therefore studied the correlation between the calculated spectral indices and the age of the blood donors. There was a slight tendency to a positive correlation for SDI recorded from healthy controls, in agreement with the previously observed PKC impairment in aged individuals (for review see Pascale et al., 2007). However, due to the high variance, this correlation was not significant. No correlation with age was found for this index within the AD population (Figure 4).

Insert Figure 5

Additionally this effect was not observed in RBC from patients suffering Parkinson's disease (PD) (Figure 5), thus making fim-1 a valuable tool that can be applied in a screening test to distinguish AD patients from healthy individuals.

What is the mechanism leading to PKC alteration in RBC from AD patients? The application of exogenous 10 μM $\text{A}\beta_{1-42}$ to RBC magnified the differences observed in SDI values between healthy controls and AD patients and PKC became insensitive to phorbol ester activation (unpublished results). Moreover we also established that PKC in RBC was not sensitive to an intracellular $[\text{Ca}^{2+}]$ increase induced by exogenous $\text{A}\beta$ application. We cannot exclude a direct effect of Ca^{2+} on calpain, which hydrolyses PKC in Ca^{2+} -insensitive PKM by cleaving the regulatory domain of the enzyme. Another hypothesis would rely on oxidative stress produced by $\text{A}\beta$ polymerization. Free radicals could oxidize SH groups on the enzyme, which would become incompetent for activation and lose its Ca^{2+} and phorbol ester sensitivity. This hypothesis should be further investigated.

These findings underline the important role of PKC in Alzheimer pathology. They also suggest a specific mechanism by which PKC plays a role not only in the central nervous system but also in peripheral organs in the development of the disease. Hence PKC appears to be a functional biomarker of Alzheimer's disease. Our results support the use of alteration in PKC conformation in red blood cells as an early possibly predictive marker for AD.

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Legend to figures

Figure 1

Interactions of PKC with APP and Tau pathways: Panel A summarizes the stimulatory (green arrows) and inhibitory (red lines) interactions involved in APP and Tau pathways. In physiological conditions (panel B) the full capacity of PKC to be activated leads to sAPP secretion. In pathogenic conditions (panel C), where PKC activity is depressed, A β production and Tau hyperphosphorylation are stimulated.

Figure 2

Spectral characteristics of Fim-1: Panel A: formula of Fim-1 (R=fluorescein). Panel B: In the presence of purified PKC the fluorescence spectrum of Fim-1 can be deconvoluted in three gaussians curves (yellow). Addition of phorbol ester (red), which induces a conformational change of the enzyme, differently affects the elementary Gaussians: the amplitude of the first Gaussian increases and the second Gaussian is shifted towards higher wavelength.

Figure 3

Spectral index values (panel A) measured using fim-1 loaded in red blood cells from AD patients (green) and healthy volunteers (red). This spectral index is proportional with the difference in the first Gaussian intensity measured in the presence of loaded fim-1 alone and after addition of staurosporine. The distribution curves of the index values (panel B) display no overlap.

Figure 4

Correlation between spectral index values and age of individuals: There is no significant correlation with age in either group (AD green and healthy volunteers red).

Figure 5

Spectral index values measured in RBC from healthy controls (n=13, red) and Parkinson's disease patients (n=15, yellow). No significant difference was observed between the two groups.

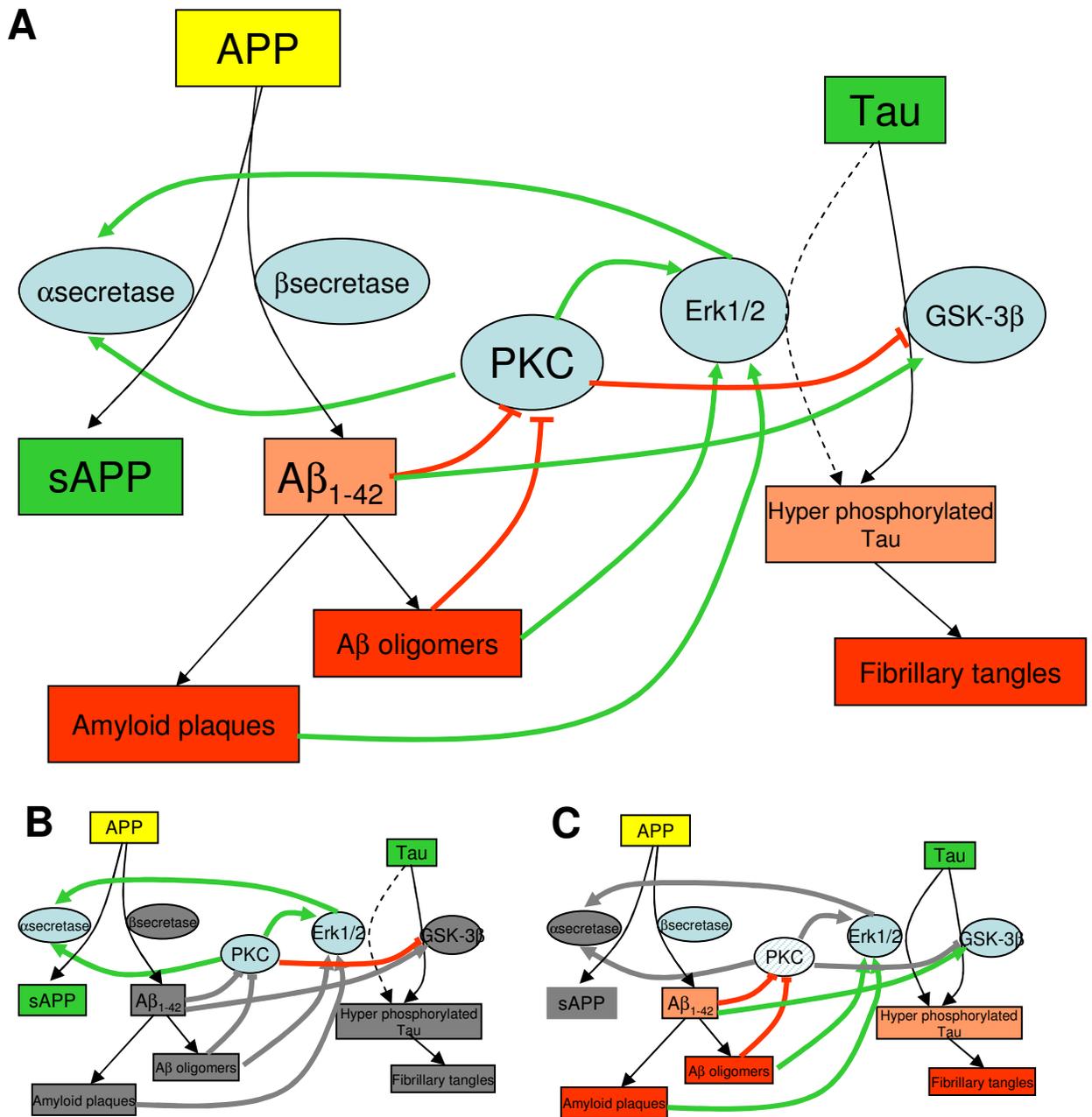


Figure 1

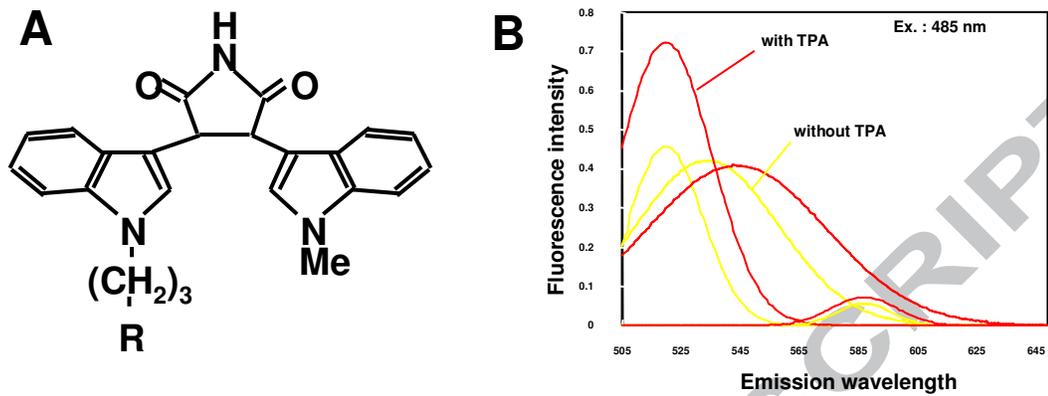


Figure 2

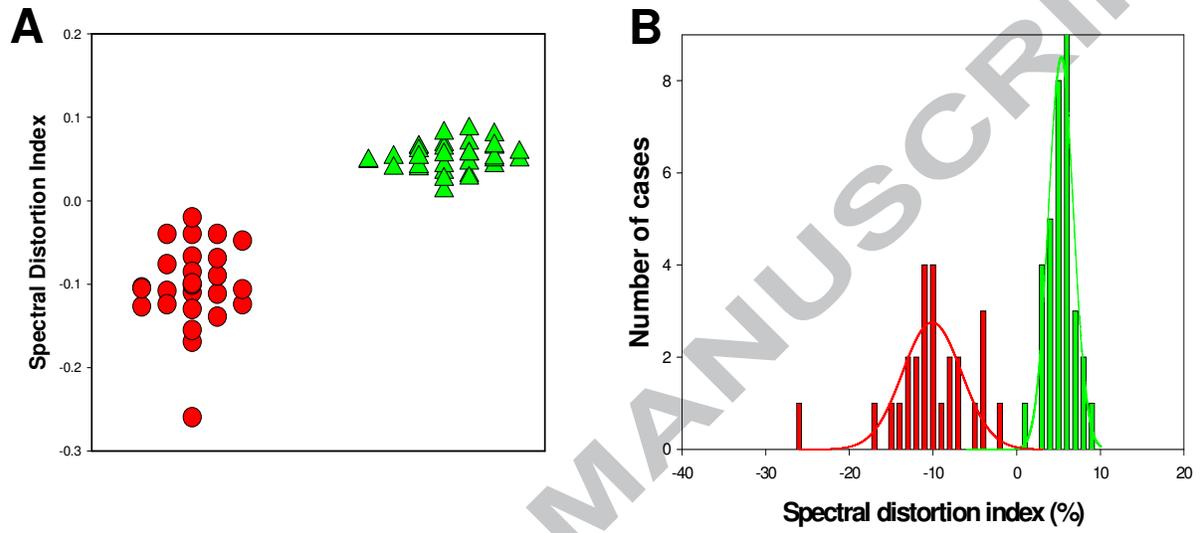


Figure 3

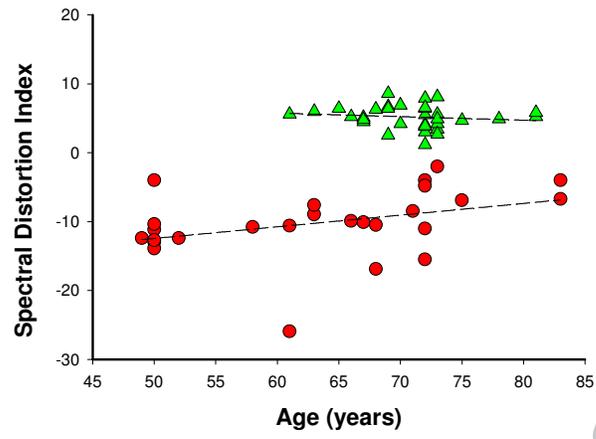


Figure 4

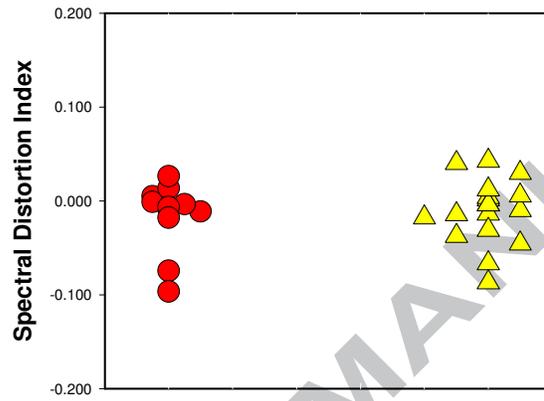


Figure 5