Use of Mass Spectrometric Approaches to Tackle Challenges in Drug Discovery: The Beta-Amyloid Paradigm

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***Abstract*— The origin of many neurodegenerative disorders like Alzheimer's Disease (AD) lies in protein processing failures, which leads to protein aggregation and accumulation as amyloid fibrils. Abnormal accumulation and aggregation of beta amyloid peptide (A*β*) eventually lead to the formation and cerebral deposition of amyloid plaques, the major pathological hallmark in AD. A*β*1-40 and A*β*1-42 are the predominant components of senile plaques formed in AD brain. The aggregation of A*β* is associated with neurodegeneration, loss of cognitive ability, and premature death. Understanding the aggregation mechanism and how to inhibit aggregate formation is therefore crucial. In light of the proposed link between oxidative stress, unregulated immune response and neurodegeneration, it is suggested that use of antioxidants may be beneficial for inhibiting A*β* fibrillogenesis. Therefore, endogenous and dietary antioxidants may offer a protective or even therapeutic alternative against amyloidosis. In this study, several compounds isolated from natural products are screened for the *in vitro* antiamyloidogenic activity. Novel electrospray ionization (ESI) mass spectrometry (MS)-based methodologies are employed to assess the noncovalent interactions between the A*β* and isolated components from natural products. The specificity and the stability of these noncovalent complexes were examined under different experimental conditions, whereas their relative binding strength was assessed. In addition, MS proteolytic mapping was employed to provide information on the noncovalent binding site of the bioactive molecule on the A*β* residues. This may shed some light into the mechanisms of AD pathology and provide insights into novel agents that can be employed towards prevention or even treatment of AD.**

Keywords*— *Alzheimer's Disease, beta amyloid peptide, electrospray mass spectrometry, natural products, noncovalent complexes

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I. INTRODUCTION

Dementia, including Alzheimer's disease (AD), is associated with a decline in memory, loss of cognitive ability and premature death, and represents one of the biggest global public health challenges. AD is the most common neurodegenerative disease worldwide accounting for 60% to 80% of senile dementia cases and it has a major impact on health along with financial ramifications in Western world. The prevalence of AD doubles every 5 years after the age of 60 years, increasing from a prevalence of 1% among those 60–64 years old up to 40% of those aged 85 years and older [1]. Even though the greatest risk factor for AD is advancing age, individuals younger than age 65 can also develop the disease. AD is the fourth main cause of death affecting more than 35 million people worldwide whereas it is projected to double in 2030, and almost quadruple until 2050 [2], whereas the number of caregivers will rise up to 216 million. Furthermore, the direct and indirect economic cost associated with the disease in 2010 was estimated at more than $600 billion worldwide, corresponding to more than 1% of the aggregated worldwide Gross Domestic product (GDP), thus making AD and other dementias the world's "18th largest economy" [3]. It should be mentioned that the average lifespan of sufferers is between 7-10 years from the time of diagnosis and no cure is presently known. In addition, most of the people suffering from AD need personal care with their needs starting early in the disease course and growing constantly over time, thus contributing to the high cost associated with the disease. Therefore, finding ways to prevent and reverse this trend is critical and represents a priority area of the European Union and most countries in the world.

At pathological level, AD is a progressive neurodegenerative disorder characterized by selective, but widespread, neuronal atrophy and degeneration, and synaptic loss in various brain regions such as hippocampus and cortex that eventually result in diffuse cerebral atrophy. The cause of AD remains ambiguous, even though many hypotheses have been proposed to enlighten its mechanism. Research over the last twenty years has revealed and clarified the pathological pathways and

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mechanisms of the disease. The prevailing hypotheses are centered on the amyloid beta peptide (Aβ) deposits and neurofibrillary tangles (NFTs) which have been described in patients with AD [4]. Even though the causes and effects associating protein aggregation and nervous system degeneration are mostly unknown, the disease-specific aggregated proteins and peptides have apparent diagnostic and even therapeutic implications [5]. The central role of the amyloid plaques and the amyloid cascade hypothesis [6] in AD pathogenesis has been confirmed by discovering AD-related polymorphisms in genes encoding the Amyloid Precursor Protein (APP) and the enzymes converting APP into amyloidogenic compounds [7]. Human AD neuritic plaques consist primarily of aggregates of Aβ peptide, which is a cleavage product of APP and it is invariably deposited in the brains of AD patients. In particular, $A\beta_{1-40}$ and $A\beta_{1-42}$ are the predominant components in amyloid plaques and they are a pathological hallmark of AD. In the process of amyloidosis, the Aβ peptide misfolds and aggregates to form an initial nucleus, comprising of a small number of Aβ molecules, followed by a rapid elongation stage after which incorporates new Aβ molecules. The main mechanisms proposed to justify Aβ's neurotoxicity are oxidative stress and unregulated inflammatory / immune response, and they are believed to play a key role for the processes inherent to AD [8, 9]. The suggested link between oxidative stress, inflammatory response and AD, justifies the use of anti-inflammatory agents as therapeutic approaches against AD [10]. On the other hand, in view of the amyloid cascade hypothesis there have been several efforts to develop therapies for AD by reducing the Aβ levels in the brain [11,12], including inhibition of amyloid formation by controlling APP proteolytic cleavage (secretase inhibitors) [13,14]. Other therapeutic alternatives include control of Aβ degradation [15], immunization with Aβ [16,17], inhibition of Aβ aggregation, and/or stimulation of its disaggregation by molecules that potentially bind on the peptide and stabilize its structure, thus becoming potential inhibitors of amyloidosis [18,19]. To date, the only FDA approved drugs for disease treatment is a group of acetylcholinesterase inhibitors: donepezil (Aricept), rivastigmine (Exelon), galantamine (Reminyl) while memantine (Ebixa) is also used in clinical practice. However, these drugs are used as symptomatic treatment and they are unable to stop or reverse the progression of the disease, not offering a definitive cure. Despite the enormous scientific progress and research effort on disease characterization and therapeutic treatment, *there is still no cure for AD*. That has emphasized the need for elucidating the AD underlying mechanisms, the availability of a drug treatment as well as the importance for diagnostic markers for AD [20] as the early symptoms of AD are rather subtle.

Plants containing antioxidants and phytoestrogens such as α-tocopherol have been employed in the management of AD [21]. It has been proposed that the plant-derived and dietary antioxidants may offer an ideal therapeutic regime for protection against the risk of the aforementioned disease. As a matter-of-fact recently standardized phytopreparation of *Gingko biloba* has been approved in some European countries for the treatment of AD [22]. Therefore, plants and their bioactive natural products are of great interest for the search of new molecules acting against AD. In particular, plants from the Mediterranean basin (a global biodiversity "hot-spot" [23], in which only the Southern part of Greece offers 6,000 plants species and 1,200 endemic [24]) are worth to be investigated. In this study, several natural compounds isolated from plants endemic in Greece, are evaluated in terms of their anti-neurodegenerative properties.

Mass Spectrometry (MS) is a powerful analytical tool for providing information on the primary structure of biomolecules, using small amounts of sample [25]. MS has played an important role in the design, discovery and development of new pharmaceuticals, from the earliest stages of disease determination through the final stages of clinical testing. The advent of electrospray ionization (ESI) and matrix-assisted laser desorption/ ionization (MALDI) methods combined with the rapid proliferation of several types of mass analyzers has revolutionized the applicability of MS for studying proteins of high molecular weights by themselves or when they are bound to ligands. That, in turn, has opened the way to elucidate macromolecular structures and study their noncovalent interactions with small molecules or other macromolecules [26], which are important aspects in understanding their biological function and developing new therapeutics. It is well known that weak noncovalent interactions can often trigger cellular functions such as those between enzyme and substrate, protein and ligand, protein and protein, antibody and antigen. Interruption of these non-covalent interactions can cause abnormalities, which often lead to diseases. Hence the elucidation of the structure and the formation mechanism of these noncovalent complexes can lead to a better understanding of the disease process and provide insight towards the design and development of a therapeutic approach [27,28].

More specifically, ESI is a mild ionization method, which allows the preservation of protein conformations and protein–ligand noncovalent interactions/associations in the gas phase [29,30]. That renders ESI MS a suitable technique for the detection of macromolecular interactions in real time [26], the definition of the stoichiometry and the topology of the interacting species, as well as the quantification of their corresponding affinities [31,32]. These noncovalent interactions between peptides/proteins and ligands may be involved in triggering cellular functions and causing diseases. This is illustrated in our efforts to screen and identify potential inhibitors for the Aβ peptide aggregation. In the present study, the noncovalent interaction between Aβ and several natural products was monitored by ESI MS. The detection of the noncovalent interactions between the Aβ peptide and the natural product/ligand may shed some light into the mechanisms of AD pathology and provide insights into the active compounds that can be employed as novel agents towards prevention or even treatment of AD.

II. MATERIALS AND METHODS

A. Sample Preparation for in vitro Screening

An aliquot of a freshly prepared Aβ (1-40) solution 100 μM (monoisotopic M_r 4327.15, average M_r 4329.9; Bachem AG,

Bubendorf, Switzerland) in deionized water was added into 150 μL of an equimolar ligand solution (100 μM) in 2 mM ammonium acetate (Merck, Darmstad, Germany) – 0.5 % acetic acid (Pancreac, Barcelona, Spain). All solvents used were of analytical grade purity. Mass spectral analysis was carried out on a Waters Premier quadrupole reflectron time-of-flight (QqTOF) and a Sciex API-III triple quadrupole instruments equipped with a standard atmospheric pressure ionization source in the positive ion mode. Oleuropein (OE) (M_r monoisotopic: 540.18) was isolated from olive leaves of *Olea europaea* (var. koroneiki), according to a previously described procedure [33] with greater than 99% purity, as it was assessed by NMR analysis. Major biologically active components of *Crocus sativus L.* were extracted, separated and isolated from dried stigmas of saffron flowers by semi-preparative HPLC as previously described [34]. Accurate mass measurements of the crocus-derived bioactive components were taken on the QqTOF Premier high-resolution MS employing micro-spray ESI. Other natural products (NP) and endogenous compounds, such as quercetin, quercetin glycoside, kaempferol and melatonin (M) were purchased and used without any prior purification.

B. Enzymatic Digestion and Mapping Analysis

Determination of the noncovalent binding site of the bioactive molecule on the A*β* peptide was carried out by tryptic and Glu-C mapping. The two different proteolytic digestion protocols were similar to the ones described in the mapping of the A*β* – M noncovalent complex [35]. The resulting peptide digests were analyzed by ESI FT-ICR MS on a Bruker Daltonics BioAPEX-94e superconducting 9.4 T FT-ICR mass spectrometer. It should be noted that the enzyme-derived peptide mixtures were desalted on a $ZipTip_{C18}$ pipette tip (Millipore, Bedford, MA, USA) prior to MS analysis using the protocol that was previously described [35].

C. Binding Strength Studies

In the analysis of the noncovalent complex between A*β* and the bioactive molecule, the mass spectrometer was tuned with gentle desolvation parameters in order to preserve noncovalent complexes intact during their transfer from solution to the gas phase. In the binding strength studies of the A*β* – M and A*β* – OE noncovalent complexes, the +5 charged ion of the noncovalent complex was selected and subjected to collision-induced dissociation (CID) in the collision cell of the API-III triple quadrupole mass spectrometer, with collision energies ranging from 5 to 20 eV.

III. RESULTS AND DISCUSSION

ESI MS is a mild ionization process, which allows the preservation of the fragile noncovalent interactions upon their transfer from aqueous solutions to the gas phase. Therefore, ESI MS represents an ideal method to provide information on the M_r of the noncovalent complexes formed in solution, as well as the stoichiometry of the interacting species. Nevertheless, it should be noted that careful optimization of the experimental parameters should be performed especially in terms of the entrance potential in the MS ion source, the ligand concentration, the pH and the presence of organic modifiers in the solution which affect conformational changes of the A*β* peptide [35]. For example, elevated values of the ion source entrance potential increases the internal energy of the complex, which in turn can induce dissociation of the gas phase noncovalent complex. The effect of the ion source entrance potential on the abundance of the noncovalent complex depends on the binding strength of the complex. Similarly, careful selection of the solution pH and the amount of the organic modifier should be made in order to avoid denaturation of the noncovalent interactions. Usually pH values lower than 4.0 and addition of an organic modifier such as methanol can lead to substantial unfolding of the peptide and eventual disruption of noncovalent interactions [35].

Screening of endogenous antioxidants, such as M or plant-derived bioactive compounds for binding to A*β* was performed by ESI MS. It has been found that only few of the compounds studied interact with A*β* via noncovalent interactions. It has been shown that A*β* interacts with M and

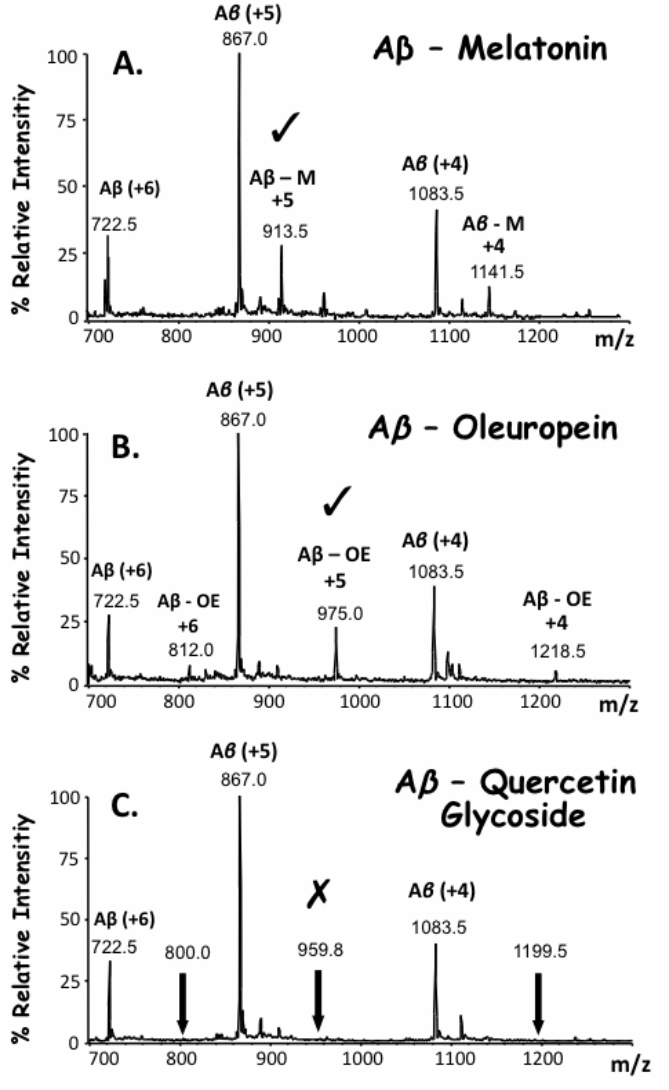

Figure 1. ESI mass spectra of an equimolar solution of the A*β* peptide with Melatonin - M (**A**), Oleuropein - OE (**B**) and quercetin glycoside (**C**).

forms noncovalent complexes with a stoichiometry varying from 1:1 to 1:2 depending on the incubation time (Figure 1A) [35]. Similar observations have been made for other plant-derived phytochemicals, such as oleuropein (OE) [36] and several metabolites thereof such as hydroxytyrosol and oleocanthal. ESI MS analysis of the Aβ – OE sample solution generated a mass spectrum containing the multiply charged ion envelope corresponding to the +4 (*m/z* 1083.5), +5 (*m/z* 867.0) and +6 (*m/z* 722.5) charge states of Aβ and a "bell-shaped" distribution of multiply charged ions at *m/z* 1218.5, 975.0, and 812.0 (Figure 1B), which corresponded to the +4, +5, and +6 charge states of the 1:1 Aβ:OE noncovalent complex, respectively.

Deconvolution of the observed ion envelopes gave rise to M_r of 4330 and 4870.0, with the latter being in good agreement with the theoretical average mass of 4870.4 for the 1:1 Aβ:OE noncovalent complex. Thus, the electrospray ionization process and the experimental conditions maintained the existing interactions in solution, thereby showing that OE forms noncovalent complexes with Aβ with 1:1 stoichiometry. The increased sensitivity of the QqTOF and the FT-ICR systems allowed the detection of low-abundance moieties such as the Met35(O)-containing $A\beta_{1\text{-}40}$ variant and the OE 1:1 noncovalent interaction thereof [37]. This is an important observation because it shows that the antioxidant OE can bind not only to Aβ but also to the already oxidized species AβMet35(O), thus showing potential use towards preventing its subsequent aggregation. Similar noncovalent interactions between the Aβ peptide and glycosylated crocetin apocarotenoids (crocins) isolated from *Crocus sativus L*. have been recently observed [38].

It is well documented that random protein–ligand interactions may occur at higher concentrations. Therefore, the specificity of the Aβ – ligand noncovalent interaction was evaluated at low concentration levels of the interacting species (5-100 μM). The occurrence of nonspecific aggregation in the gas phase can be avoided by reducing the concentration of the interacting species. In our case, the ESI signals corresponding to the 1:1 Aβ – OE and Aβ – M noncovalent complexes were present for all concentration levels, thus indicating a very specific interaction. On the contrary, ESI MS analysis of the Aβ – quercetin glycoside solution yielded no signals corresponding to the formation of a noncovalent complex (Figure 1C); thus showing the specificity of the previously observed interactions of the Aβ peptide with OE and M.

The stability of the noncovalent complexes was studied under several experimental parameters, such as elevated ion source entrance potential, low pH values and the presence of organic modifier. The overall tested conditions of the ESI process do not perturb the interactions that govern the complex formation of Aβ with M and especially with OE, with the latter showing considerable stability even under experimental conditions, which usually do not favor noncovalent interactions (e.g., high organic modifier content). It should be noted that addition of 45% MeOH yielded the most abundant signals associated with the complex, whereas the Aβ – OE noncovalent complex was preserved even at higher MeOH content although the observed ESI signals had lower S/N. The preservation of the Aβ – OE noncovalent complex at these conditions reflects the high binding strength of this interaction.

The binding strength of the Aβ – ligand complex was first examined under collisional excitation in the declustering region of the mass spectrometer. Thus, the effect of the ion source entrance potential on the abundance of the noncovalent complex was studied at entrance potential values ranging from 35 to 100 V. That yielded a qualitative assessment of the binding energy of the Aβ – ligand noncovalent complexes, with the ones corresponding to OE and some crocins presenting the highest binding affinities. Moreover, binding strength studies of the Aβ – M and Aβ – OE noncovalent complexes were performed by ESI tandem MS analysis (Figure 2). In this study the +5 charged ion of the noncovalent complex was selected and subjected to CID in the collision cell of a triple quadrupole mass spectrometer at collision energies ranging from 5 to 20 eV. It is clearly shown that it takes more energy to dissociate the Aβ – OE signal than that of the Aβ – M, thus demonstrating the higher binding strength of the Aβ – OE noncovalent complex over that of the Aβ – M counterpart.

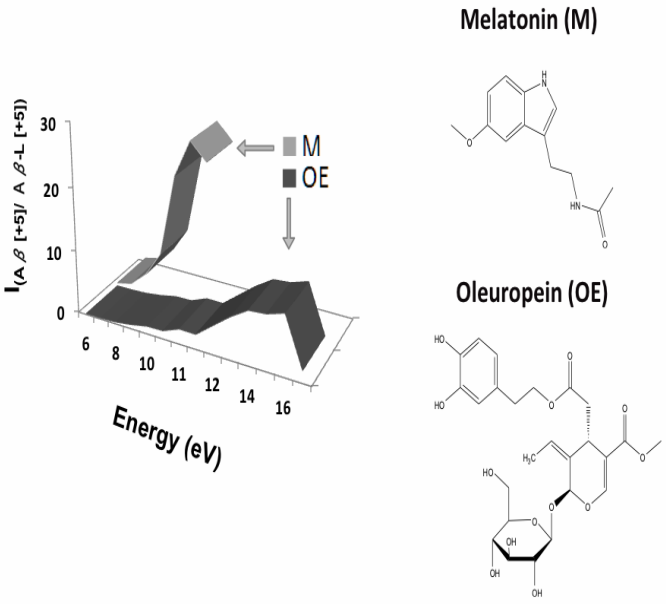

Figure 2. Binding strength studies of the Aβ – M and Aβ – OE noncovalent complexes as demonstrated by the MS/MS dissociation curves of the +5 charged ion of the associated noncovalent complex.

Concerning the binding site of the ligands on Aβ, two different proteolytic digestion protocols with trypsin and Glu-C protease have been employed combined with ESI FT-ICR MS analysis of the resulting proteolytic fragments. In case of the M and OE ligands, the resulting mass spectral data indicate that the [4-11] Aβ amino acid sequence and the [17-28] hydrophobic region of Aβ are responsible for the noncovalent interaction [37]. This mapping study revealed that the

interacting region of Aβ with M and OE lies within the hydrophobic region of the peptide, which is also responsible for the β-sheet conformation in aqueous solutions and consequently its aggregation. It has also been reported that the [17-21] hydrophobic area may play a controlling role in fibrillogenesis [39]. This proteolytic MS mapping information on the binding site could be used to reveal the specific residues that are involved in Aβ's interaction and fibrillation process.

Following the initial ESI MS screening of the inhibitory ability of the bioactive compounds against Aβ aggregation, *in vitro* scanning of the compounds of interest towards the inhibition of APP misprocessing and Aβ generation, as well as the abnormal tau hyperphosphorylation, will be performed on different human and mouse neuronal cell lines. These assays will provide input for the selective and targeted selection of the most active molecules in terms of anti-amyloidogenic activity.

IV. CONCLUSION

This study demonstrates the excellent suitability of ESI MS for studying noncovalent interactions between Aβ and biologically active components isolated from NPs. The presented ESI MS-based methodology may serve as a screening tool for evaluating NPs and structural analogues thereof, thus facilitating the design of novel potential anti-amyloidogenic agents. Moreover, the *in vitro* screening using MS-based approaches is an excellent preliminary step for the subsequent evaluation stage by cell viability assays involving different human and mouse neuronal cell lines. The successful detection of these noncovalent complexes of Aβ with NPs could be invaluable in the area of neuroscience research. This methodology could allow real-time monitoring of the aforementioned interactions, thus shedding some light into the mechanisms of AD pathology and facilitating the design and development of novel compounds, which could act as protective or even therapeutic agents against AD. These NPs and derivatives thereof can be eventually exploited, in the form of nutraceuticals and/or drug formulations towards the prevention and/or treatment of AD.

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REFERENCES

[1] E. von Strauss, M. Viitanen, D. De Ronchi, B. Winblad, and L. Fratiglioni, "Aging and the occurrence of dementia: Findings from a population-based cohort with a large sample of nonagenarians," *Arch Neurol* 1999, 56, 587–592.

[2] World Alzheimer's Report 2013.

[3] Karolinska Institute Report on Dementia 2010.

[4] C. Behl, "Alzheimer's disease and oxidative stress: implications for novel therapeutic approaches," *Prog. Neurobiol.* 1999, 57, 301-323.

[5] C.A. Ross and M.A. Poirier, "Protein aggregation and neurodegenerative disease," *Nat. Med.* 2004, 10 (Suppl), S10-S17.

[6] J.A. Hardy and G.A. Higgins, "Alzheimer's disease: the amyloid cascade hypothesis," *Science* 1992, 256, 184-185.

[7] J. Hardy, "Has the amyloid cascade hypothesis for Alzheimer's disease been proved?", *Curr. Alzheimer Res.* 2006, 3, 71-73.

[8] W. Retz, W. Gsell, G. Munch, M. Rosler and P. Riederer, "Free radicals in Alzheimer's disease," *J. Neural Transm.* 1998, 54, 221-236.

[9] G. Aliev, M.A. Smith, D. Seyidov, M.L. Neal, B.T. Lamb, A. Nunomura, E.K. Gasimov, H.V. Vinters, G. Perry, J.C. LaManna, and R.P. Friedland, "The Role of Oxidative Stress in the Pathophysiology of Cerebrovascular Lesions in Alzheimer's Disease," *Brain Pathol.* 2002, 12, 21-35.

[10] P.L. McGeer, M. Schulzer, and E.G. McGeer, "Arthritis and Anti-Inflammatory Agents as Possible Protective Factors for Alzheimer's Disease: A Review of 17 Epidemiologic Studies," *Neurology* 1996, 47, 425–432.

[11] E. Giacobini and G. Gold, "One hundred years after discovery of Alzheimer's disease. A turning point for therapy?" *J. Alzheimers Dis.* 2007, 12, 37–52.

[12] R.E. Becker and H.H. Greig, "Increasing the success rate for Alzheimer's disease drug discovery and development," *Expert Opin. Drug Discov.* 2012, 4, 367–370.

[13] J. Walter, and C. Haass, "Secretases as Targets for β-Amyloid Lowering Drugs," *Drug Dev. Res.* 2002, 56, 201–210.

[14] D.M. Walsh, I. Klyubin, J.V. Fadeeva, M.J. Rowan, and D.J. Selkoe, "Amyloid- β Oligomers: Their Production, Toxicity, and Therapeutic Inhibition," *Biochem. Soc. Trans.* 2002, 30, 552–557.

[15] V. Chesneau, K. Verkellis, M.R. Rosner, and D.J. Selkoe, "Purified Recombinant Insulin-Degrading Enzyme Degrades Amyloid β-Protein but Does Not Promote Its Oligomerization," *Biochem. J.* 2000, 351, 509–516.

[16] D.K. Lahiri, M.R. Farlow, N.H. Greig, and K. Sambamurti, "Current Drug Targets for Alzheimer's Disease Treatment," *Drug. Dev. Res.* 2002, 56, 267–281.

[17] D.J. Selkoe, "Deciphering the Genesis and Fate of Amyloid β –Protein Yields Novel Therapies for Alzheimer Disease," *J. Clin. Invest.* 2002, 110, 1375–1381.

[18] P.T. Lansbury Jr., "Back to the Future: The Old-Fashioned Way to New Medications for Neurodegeneration," *Nat. Rev. Neurosci.* 2004, 5, S51–S57.

[19] P.T. Lansbury Jr., "Following Nature's Anti-Amyloid Strategy," *Nat. Biotechnol.* 2001, 19, 112–113.

[20] S.T. DeKosky and K. Marek "Looking backward to move forward: Early detection of neurodegenerative disorders," *Science* 2003, 302, 830–834.

[21] M.J.R. Howes, N.S.L. Perry, and P. Houghton, "Plants with Traditional Uses and Activities Relevant to the Management of Alzheimer's Disease and Other Cognitive Disorders," *J. Phytother. Res.* 2003, 17, 1–18.

[22] M.M. Essa et al. "Neuroprotective Effect of Natural Products Against Alzheimer's Disease." *Neurochem. Res.,* 2012, 37, 1829-1842.

[23] N. Myers, R.A. Mittermeier, C.G. Mittermeier, G.A. da Fonseca, and J. Kent, "Biodiversity hotspots for conservation priorities," *Nature* 2000, 403, 853-858.

[24] W.V. Reid, "Biodiversity hotspots," *Trends Ecol. Evol.,* 1998, 13, 275-280.

[25] K. Biemann and S.A. Martin, "Mass spectrometric determination of the amino acid sequence of peptides and proteins," *Mass Spectrom. Rev.* 1987, 6, 1-76.

[26] J.A. Loo, "Electrospray Ionization Mass Spectrometry: A Technology for Studying Noncovalent Macromolecular Complexes," *Int. J. Mass Spectrom.* 2000, 200, 175–186.

[27] A. Ganguly, B.N. Pramanik, G. Chen and A. Tsarbopoulos, "Detection of Non-covalent Complexes by Electrospray Ionization Mass Spectrometry" in *Applied Electrospray Mass Spectrometry*, Marcel Dekker: New York, 2002; p. 361-387.

[28] B.N. Pramanik, P.L. Bartner, U.A. Mirza, Y.H. Liu and A.K. Ganguly, "Electrospray Ionization Mass Spectrometry for the Study of Noncovalent Complexes: an Emerging Technology," *J. Mass Spectrom.* 1998, 33, 911-920.

[29] B. Ganem, Y.T. Li, and J.D. Henion, "Detection of Noncovalent Receptor Ligand Complexes by Mass-Spectrometry," *J. Am. Chem. Soc.* 1991, 113, 6294–6296.

[30] A.K. Ganguly, B.N. Pramanik, A. Tsarbopoulos, T.R. Covey, E. Huang and S.A. Fuhrman, "Mass Spectrometric Detection of the Noncovalent GDP-bound Conformational State of the Human H-Ras Protein", *J. Am. Chem. Soc.* 1992, 114, 6559-6560.

[31] S. Mathur, M. Badertscher, M. Scott and R. Zenobi, "Critical Evaluation of Mass Spectrometric Measurement of Dissociation Constants: Accuracy and Cross-validation against Surface Plasmon Resonance and Circular Dichroism for the Calmodulin-Melittin System," *Phys. Chem. Phys*. 2007, 9, 6187–6198.

[32] A. Wortmann, M.C. Jecklin, D. Touboul, M. Badertscher and R. Zenobi, "Binding Constant Determination of High-Affinity Protein–Ligand Complexes by Electrospray Ionization Mass Spectrometry and Ligand Competition," *J. Mass Spectrom.* 2008, 43, 600–608.

[33] B. Shasha, J. Leibowityz, "On the Oleuropein, the Bitter Principle of Olives," *J. Org. Chem.* 1961, 26, 1948–1954.

[34] N.S. Koulakiotis, E. Pittenauer, M. Halabalaki, A. Tsarbopoulos, G. Allmaier, "Comparison of different tandem mass spectrometric techniques (ESI-IT, ESI- and IP-MALDI-QRTOF and VMALDI-TOF/RTOF) for the analysis of crocins and picrocrocin from the stigmas of *Crocus sativus* L.," *Rapid Commun. Mass Spectrom.* 2012, 26, 670-678.

[35] F.N. Bazoti, A. Tsarbopoulos, K. Markides and J. Bergquist, "Study of the Non-Covalent Interaction between Amyloid-β Peptide and Melatonin using Electrospray Ionization Mass Spectrometry," *J. Mass Spectrom.* 2005, 40, 182-192.

[36] F.N. Bazoti, J. Bergquist, K. Markides and A. Tsarbopoulos, "Detection of the Non-Covalent Complex between Amyloid-β Peptide (1-40) and Oleuropein using Electrospray Ionization Mass Spectrometry," *J. Am. Soc. Mass Spectrom.* 2006, 17, 568-575.

[37] F.N. Bazoti, J. Bergquist, K. Markides and A. Tsarbopoulos, "Localization of the Binding Site in the Non-Covalent Interaction between Amyloid-β Peptide (1-40) and Oleuropein Using Electrospray Ionization FTICR Mass Spectrometry," *J. Am. Soc. Mass Spectrom.* 2008, 19, 1078-1085.

[38] N.S. Koulakiotis, D. Anagnostopoulos and A. Tsarbopoulos, "Screening of *Crocus sativus* L.-derived components for noncovalent interaction with amyloid-β peptide (1-40) using Electrospray Ionization Mass Spectrometry," unpublished results.

[39] D.M. Walsh, A. Lomakin, G.B. Benedek, M.N. Condron, D.B. Teplow, "Amyloid Beta-Protein Fibrillogenesis. Detection of a Protofibrillar Intermediate," *J. Biol. Chem.* 1997, 272, 22364–22372.

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