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Natural product polyphenol inhibition of amyloid-β aggregation

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Abstract

β-amyloid peptide (Aβ) aggregation has been hypothesized to be fundamentally involved in the development of Alzheimer's disease (AD), a chronic neurodegenerative disorder. The effects of this aggregation are exacerbated by the interaction of Aβ with the misfolded cellular human prion protein, both of which constitute amyloidogenic plaques. There is a direct correlation between β-amyloid protein aggregation and uncontrolled neural cell apoptosis, preludial to a majority of Alzheimer's symptoms. Assuming causation, a potential preventative and curative therapy for Alzheimer's and related neurodegenerative diseases could be based on the inhibition of β-amyloid and related prion protein aggregation. Polyphenols are naturally occurring organic molecules that consist of one or more aromatic phenolic rings. The methods described in this paper can be used to identify the most effective natural product polyphenols inhibitors. This paper discusses various *in vitro* and *in vivo* assays; the *in vivo* methods utilize *C. elegans*, which is an ideal model organism due to the similarities it shares with human biological characteristics and as its short lifespan.

Keywords

Polyphenols, Natural Products, Amyloid-β, Alzheimer's Disease, Neurodegenerative Disorders, Prion protein

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Background

Amyloid-β

Neurodegenerative diseases, including Alzheimer's, have long been the subject of research. There is a direct correlation between β-amyloid proteins and uncontrolled neural cell apoptosis, preludial to a majority of Alzheimer's symptoms (1-2). Amyloid-associated disorders are associated with fibrillar protein deposits, which may contribute to the cytotoxicity of β-amyloid proteins (3). A potential preventative and curative therapy to Alzheimer's and related neurodegenerative diseases could be based on the inhibition of β-amyloid proteins and related prion proteins. In 1907, Aloysius Alzheimer used silver-staining to examine the brain of Auguste D, one of his patients. The novel technique allowed Alzheimer to find neurofibrillary tangles and other plaques such as Aβ or tau, which are the hallmarks of the disease. Although the exact etiology of the disease is unknown, there have been great strides made over the past century in our understanding of the disease (1).

Toward the end of the 1990s, mutations in three genes (the presenilin 1 gene on chromosome 14, the amyloid precursor gene on chromosome 21, and the presenilin 2 gene on chromosome 1) were identified as risk factors for early-onset AD. However, these genes are only responsible for < 2% of all cases for AD. There is a more common risk factor for late-onset of the disease, type ε4 of the gene responsible for apolipoprotein E (APOE). This genetic marker is unlike the those mentioned above as "the APOE ε4 allele is not deterministic, but confers an approximately three-fold risk of developing AD if one copy of the ε4 allele is present, and an eight-fold risk if two copies are present" (2). While scientists have been unable to determine the cause behind Alzheimer's, the most influential hypothesis is that of the amyloid cascade: deposits of amyloid- β are responsible. This is supported by large buildups of amyloid and tau proteins in the brains of individuals with Alzheimer's disease. The accumulation of the amyloid-β protein (or amyloid-β plaques) outside neurons and the accumulation of an abnormal form of the tau protein (or tau tangles) inside neurons are two of several pathologies believed to contribute to the damage and destruction of neurons that, in turn, result in memory loss, dementia, mood swings, and the other symptoms of Alzheimer's. The accumulation of amyloid-β and tau tangles are two neuropathological indicators of Alzheimer's Disease (3-6). Despite its widespread popularity, there have been several studies that contradict the amyloid-cascade hypothesis. Studies have shown that in some individuals, neurodegeneration can occur before the onset of amyloidogenesis, and that amyloidogenesis is sometimes observed in the absence of Alzheimer's disease. Further research suggests that many individuals with preclinical AD do not follow the proposed temporal order of the amyloid-cascade hypothesis (2). Other theories, such as the A/T/N system, still acknowledge the role of amyloid-β and tau proteins, but refute causal relationships suggested by the amyloid-cascade hypothesis.

Multiple mechanisms have been proposed for amyloid cytotoxicity. The primary theory in modern research hypothesizes that amyloid forms pores in neural cell membranes, destabilizing and permeating the neural cells and interfering with synaptic and vital intracellular functioning. This results in increased levels of reactive oxygen and nitrogen species as a result of cell redox system malfunctions, aggregation causing protein lack of function, and hyper-phosphorylation of

proteins, all leading to aggregative protein deposits (3). β-amyloid protein aggregation is caused by the cellular prion protein, PrPC, being malconverted into PrPSc (scrapie prion protein). Figure 1 shows the formation of Aβ plaques. While PrPC is protease-sensitive and therefore regulated, PrPSc is an oligomeric form of this prion, and therefore protease-resistant (4). PrPSc binds to the β-amyloid cell surface and "exhibits dramatic lateral mobility inhibition," allowing for the cytotoxic symptoms that are associated with β-amyloid proteins (5).

PrPC is spontaneously converted to PrPSc by mutations that affect structure and thermodynamics, or allow for spontaneity of the conversion. The spontaneity is proposed to be caused by structural disruptions to the hydrophobic core (6). This conversion "involves major refolding of the C-terminal α-helical region" (7). The C-terminal helix 3 is three-dimensionally swapped and the disulfide bonds are rearranged, while a two-stranded antiparallel β-sheet interchain is formed at the "dimer interface by residues located in helix 2 in the momeric NMR structures." This dimerization occurs during a crystallization process, a process that takes several weeks to complete. The process occurs in equilibrium, with dimerized proteins selectively crystallizing and initiating further covalent dimer formation. While PrPC is largely α-helical, PrPSc is mainly composed of β-sheets (8).

Tau proteins

Tau proteins stabilize and provide flexibility to neural microtubules (9). The microtubule-associated protein tau, MAPT, is alternatively spliced to produce these proteins (10). The proteins work with tubulin, a globular protein, aiding in the assembly of the tubulin in the microtubules. Their malfunction is also associated with nervous system pathologies like Alzheimer's. Figure 2 demonstrates the relationship between tau proteins and Aβ. Tau is "preferentially located in axons rather than dendrites or cell bodies," however, when diagnosed with Alzheimer's, a brain will present tau in abnormal neurofibrillary tangles. Tau proteins rely on isoforms and phosphorylation to control microtubule stability; the hyperphosphorylation (three to four times the normal) of these tau proteins "can cause the helical and straight filaments to tangle," and these tangles are symptomatic of Alzheimer's (11-12). There are six isoforms of tau, and generally, brains diagnosed with Alzheimer's show hyperphosphorylation of all six of these isoforms in paired helical filaments. Tau mutations specifically found in frontotemporal dementia encourage the excessive hyperphosphorylation of tau. An "exosome-based mechanism" could cause tau protein release in Alzheimer's (13).

Only a section of the microtubule-binding domain is necessary for the polymerization of the tau protein. The C terminus of the tau protein has been found to "inhibit the assembly process," and this inhibition could potentially be reversed in part using "site-specific phosphorylation" and wholly reversed by "truncation events at various sites from S(320) to the end of the molecule."

Prion protein

Prion diseases, or transmissible spongiform encephalopathies (TSEs), are caused by structural abnormalities in naturally-occurring proteins. Human TSEs include Creutzfeldt–Jakob Disease, Gerstmann–Sträussler–Scheinker syndrome, and fatal familial insomnia. Other mammals such as goats, sheep, and cows suffer from scrapie and bovine spongiform encephalopathy (14). There are three forms of prion diseases; sporadic, acquired, and genetic. The origin of sporadic prion diseases is currently unknown, but point mutations in the prion gene are responsible for the genetic forms. Acquired prion diseases are rare, typically occurring when healthy organisms with PrPC are exposed to biological material containing PrPSc (15).

The mechanism behind prion toxicity is unknown, but several theories exist to explain this phenomenon. One widely agreed upon theory is that the conformational conversion of PrPC to PrPSc plays a central role in neurodegeneration in prion diseases (16-17). There are three main hypotheses that stem from this idea. The first is that PrPSc has novel toxic properties that PrPC does not possess, also referred to as the toxic gain of function mechanism. Aggregates of PrPSc may trigger apoptotic pathways or interfere with synaptic function. Alternatively, there exists the loss of function hypothesis. This theory states that PrPC possesses a particular biological function that is lost upon the conversion to PrPSc, causing the neurodegeneration characteristic of AD that is otherwise attributed to amyloid β aggregation. The third theory suggests that PrPC has a neuroprotective effect, which then becomes a neurotoxic effect, as a result of interaction with PrPSc (18).

Polyphenol Mechanisms of action

Polyphenols are a large family of naturally occurring organic compounds that possess one or more aromatic phenolic rings. They are a well-known, class of phytochemicals spanning a diverse variety of plants and plant products, including commonly consumed products such as berries, wine, cocoa, nuts and tea. Their natural function is typically to provide protective antioxidant effects against disease, UV light, and other potentially cytotoxic and oxidizing factors (3). They can be further subdivided based on the number and arrangement of phenolic rings they possess, with the majority falling into four categories: flavonoids, phenolic acids, stilbenes, and lignans. Flavonoids have a common basic structure of two carbon rings joined by a three carbon heterocycle that includes oxygen. Common examples include quercetin, myricetin, and catechins. Phenolic acids are typically derivatives of hydroxybenzoic acid or hydroxycinnamic acid, and common examples are ferulic acid and caffeic acid. Stilbenes possess two phenyl groups joined by a two carbon bridge, and a very well studied example is resveratrol, which occurs commonly in grapes. Finally, lignans include a 2,3-dibenzyl butane structure as their hallmark, an example is secoisolariciresinol, which is commonly found in linseed (19).

In the context of AD, polyphenols' antioxidant activity has varied applications. Possible locations of polyphenolic inhibition can be seen in Figure 3. At a molecular level, the phenolic rings form π-stacking interactions with aromatic residues of amyloid fibrils (20). These residues are a common component of amyloid fibrils; it has been reported in human muscle acylphosphatase aggregation that substitutions of aggregation-promoting residues with non-aromatic ones resulted in decreases in

aggregation rate (21). Furthermore, aromatic side chains in the Aβ peptide, specifically at position 19, are strongly linked to the nucleation phase of aggregation (22). Congo Red, a dye used to stain amyloid fibrils, binds to the phenylalanine24 residue with strong stacking interactions, has been shown to inhibit insulin fibril formation (23). Individually, polyphenols vary at the specific time point of inhibition, with myricetin inhibiting nucleation, morin and datiscetin inhibiting nucleation and elongation, curcumin, quercetin, and kaempferol inhibiting elongation, and epigallocatechin gallate (EGCG) and gallic acid inhibiting elongation and redirecting the fibrillation pathway (24). Figure 4 shows an example of stacking interactions between phenolic rings.

At a cellular level, amyloid plaques are strongly cytotoxic. One major deleterious effect produced by the accumulation of amyloid plaques outside cells is the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (3). A wide variety of polyphenols mitigate this oxidative stress through metal ion chelation, by their innate free radical scavenging properties, or by favorable interactions with the enzymes that compose the body's natural antioxidant mechanisms, such as catalase. Extracts from the Gingko Biloba plant exhibit these mechanisms, where the flavonoid fraction was found to be responsible for improved survival and inhibition of ROS and RNS accumulation in rat hippocampal cell culture (26). Curcumin, a polyphenol derived from the turmeric plant Curcuma Longa, which is commonly used as a curry spice, has also been reported to possess strong antioxidant properties, with Western blot analysis confirming decreased oxidized protein presence in the residual and piriform cortices of mice (27). Metal ion chelation is another promising area for investigating polyphenolic activity, and quercetin, rutin, and (+) catechin show strongly antioxidant properties as a result of strong chelation of the Fe(II) ion, with lesser but still present chelation of the Zn ion, and only minor effects on the Cu(II) ion (28). These ions, particularly Cu(II), play a major role in the production of free radical species, as they form complexes with Aβ as it aggregates, which is believed to catalyze the reduction of diatomic oxygen, triggering a cascade of free radical production ending with the production of the extremely active hydroxyl (·OH) radical with well-known deleterious effects on cell functioning and health (29). The last major mechanism of polyphenol action is upregulation of beneficial antioxidant enzymes such as catalase, superoxide dismutase (SOD), and glutathione peroxidase. Polyphenols, as inhibitors of amyloid-β peptide fibrillation, have faced challenges with bioactivity as they often quickly degrade into inactive metabolites. However, a study where participants ate a phenolic deficient diet accompanied by 300 mL of red wine, known to be rich in flavonoid polyphenols such as resveratrol, found that SOD gene expression increased during one week of the diet, making this another avenue of research to explore (30).

Three specific polyphenols that have been widely studied will be further discussed: curcumin, green tea catechins, and resveratrol. The structures of these polyphenols are shown in Figure 5.

Curcumin

Curcumin has a wide variety of beneficial mechanisms against AD (31) . It strongly inhibits Aβ peptide, decreasing the formation of Aβ oligomers at lower doses or even leading to disaggregation and decrease in plaque size, as shown by ELISA results with an IC50 of

0.81-1.0 μM. This may be the result of its similarity to Congo Red, a confirmed amyloid-β inhibitor (32). Curcumin may have a putative activity in inhibiting the metabolism of amyloid precursor protein (APP), since structurally similar compounds pyrazole and isoxazole derivatives exhibited this property (33). It is also a metal-chelator. Its affinity to Cu(II) ions was quantified by spectrophotometry and showed that most copper ions would bind at least two curcumin molecules, reaching a half maximum at 3-12 μM copper (34). Curcumin's poor bioavailability, degrading readily in PBS at pH = 9.0 and 80℃ in 20 mins, poses an obstacle for use as a treatment, but its degradation products were also found to be effective, demonstrating lower IC50 values than predicted in vitro based on docking calculations (35). Overall, curcumin and its derivatives are highly promising as potential Aβ inhibitors.

Green Tea Catechins

Green tea is significant in many cultures for its purported anti-aging properties and other health benefits, specifically polyphenolic catechins, which have significant anti-AD potential. In the context of the prion hypothesis , multiple members of the family were found to be effective PrPSc inhibitors, including epigallocatechin (EGCG), epicatechin, and gallic acid, with the most effective, 2′,2‴-bisepigallocatechin digallate (2′,2‴-BGCD) with an IC50 of about 100 nM (36). EGCG acts on the APP metabolism pathway, changing the enzyme that cleaves APP to the non-amyloidogenic α-secretase, with mice treated with EGCG showing reduced amounts of APP levels. Furthermore, EGCG also has strong iron chelating properties, with "The two points of attachment of transition metal ions to the flavonoids molecule [being]: the o-diphenolic groups in the 3′,4′-dihydroxy positions in the B ring, and the keto structure 4-keto,3-hyroxy or 4-keto and 5-hydroxy in the C ring of the flavonols" (37). Studies on Japanese and Chinese elderly (>70 years) populations found that drinking more than two cups of tea per day resulted in a statistically significant lower level of cognitive impairment, most likely a result of the EGCG, as catechins make up more than 30% of the dry mass of a tea leaf (38). EGCG and its accompanying family, the green tea catechins, pose a potentially productive area of research for further exploration into their various mechanisms and neuroprotective effects as a possible AD treatment.

Resveratrol

Resveratrol, or 3,5,4'-trihydroxy-trans-stilbene, is a polyphenol commonly found in grapes and berries, and most recognizably in red wine with a variety of other similar molecules such as piceatannol and pterostilbene (39). It has some anti-Aβ properties, as it was found to bind to the N-terminus, at residues 5-20, of the Aβ1-42 monomer, capping the maximum length and preventing further oligomerization beyond 2 nm (40). Additionally, it has been shown to possess strong anti-inflammatory effects against Aβ triggered microglial inflammation in PC12 cells, and showed a strong neuroprotective effect as a result of "inhibiting the TLR4/NF-κB/STAT signaling cascade" (41). In the nematode, Caenorhabditis Elegans, specifically in the CL2006 strain, which expresses transgenic amyloid-β that inhibits a specific muscle promoter, resveratrol at 100 μM was found to reduce paralysis by 40%, a result of posited increases in proteasomal degradation and autophagy activity (42). It also plays a significant role as an activator of Aβ clearing enzymes such as neprilysin, where an increase in activity was confirmed through thiorphan-sensitive enzymatic assays on intact

HEK293 cells. Resveratrol appears to have an overall positive effect in increasing proteasome activity (43). In mice, based on the Morris Water Maze Test, resveratrol resulted in higher spatial memory in AD phenotype mice compared to controls, and based on Thioflavin S staining from mouse brain sections, resulted in a decrease in the number of plaques in vivo; however, evidence on whether this was a result of activity on SIRT1 and subsequent epigenetic regulation of the APP pathway is contradictory, with some results pointing to a decrease in APP, sAPPα, sAPPβ and BACE1 based on western blot analysis (44).

Overall, Porat et. al. sums the role of polyphenol inhibitors most specifically and concisely as "These structural similarities [of a common phenolic ring] imply for three-dimensional conformations that are essential for the non-covalent interaction with β-sheet structures, which are common to all amyloidogenic structures." (3). Polyphenols represent a significantly promising category of naturally occurring potential drugs.

Methods to determine polyphenol efficacy in a *C. Elegans* model

Thioflavin T

Thioflavin T (ThT) is a benzothiazole dye commonly used to assess the existence of amyloid fibrils, both in vitro and in vivo. The dye exhibits increased fluorescence, due to micelle formation, when bound to amyloid fibrils (45). Specifically, the dye's excitation (385 nm to 450 nm) and emission (445 nm to 482 nm) maxima dramatically increase, resulting in a bright yellow-green fluorescence. Furthermore, the binding of the dye is associated with the presence of cross-β structure in the fibrils (46). Any results obtained must be verified with the use of Congo Red or through electron microscopy. There are several advantages to using Thioflavin T, one of which is that the intensity of the fluorescence is linearly dependent on the fibril concentration, for a given fibril type and constant concentration of dye. Secondly, Thioflavin T does not affect the kinetics of amyloid fibril formation in vitro. The use of the dye also has some drawbacks. Thioflavin T is not specific to amyloid fibrils and can detect the presence of other structures such as fibrin and keratin. The emission and interaction of the dye with fibrils is affected by the ionic strength, pH, and viscosity of the buffer. All of these factors must be considered when determining changes in fibril concentration. Lastly, using the dye to assess the use of small molecules as inhibitors is not straightforward. Some small molecules have structures similar to that of Thioflavin T, possibly resulting in their binding to the fibrils via competitive inhibition or spectral overlap that overpowers the dye's signals rather than inhibiting the formation of fibrils (47). The use of Thioflavin T is useful due to its ease of availability and use. However, any results should be verified through the use of other tools.

Congo Red

Congo Red is an azo dye that is used to identify amyloids in vitro as it binds to several amyloid-β proteins (48). The binding of Congo Red to amyloid proteins is related to the β pleated sheet structure, as the dye binds to sites antiparallel to the β sheet on fibrils and protofibrils, which allows for the structure of the aggregates to be observed. The absorption spectrum for the dye is dependent on pH, as it has been observed that in acidic conditions, the dye has maximum absorption and transmits blue and violet wavelengths (48). However, there are certain drawbacks with the use of Congo Red, such as toxicity and its nonspecific

nature (49). Although Congo Red alone is not effective in studying amyloid protein aggregates due to a significant number of false positives, the assay, when combined with the Thioflavin-T assay allows for determining fluorescence measurements and the emission and excitation spectrum. By performing the assay with both dyes, binding competition and efficiency between the dyes and the protein can be determined (50). The excitation and emission wavelengths for Thioflavin-T and Congo Red are 450 nm and 610 nm respectively (51). The assay monitors the changes in fluorescence of the ThT and the changes in absorbance of the Congo Red by utilizing both dyes simultaneously instead of performing the assay for each dye separately. The combined assay can clearly reveal the structures and aggregation of the proteins (52). Congo Red has been observed to cause interference in the protein aggregation processes and the fibrillization of amyloid-β proteins, indicating that it can be used as a treatment to prevent diseases such as Alzheimer's (48).

Turbidity

By measuring the incident light dispersed perpendicular to the sample, the concentration of particulate matter in a sample of solution can be determined (53). This test allows observation of the growth of amyloid-β-protein aggregation in the presence of various polyphenols. The objective of performing a turbidity assay is to identify which polyphenol is most efficient in reducing the scattering by preventing amyloid-β protein aggregation. The amyloid-β protein specifically has traits that allow it to possess "intrinsic light scattering properties" (53). By measuring the light scattered throughout the solution, the amount of protein aggregate formed can be determined. The protein aggregate is in the form of microtubules and other actin filaments (54). An increase or decrease of these insoluble protein particles determines the incident light that is scattered when passed through the solution. Hence, there is a direct relationship between the solution's turbidity and the light scattered (55). The assay is performed using a microplate reader that records the concentrations of the solutions at different time intervals. For the specific polyphenols pertinent to the research focused on in this paper, a 96 microplate reader that can take optical density measurements at 600 nm was found to be ideal.

Chemotaxis

C. elegans are among the many animals which utilize chemotaxis as a tool to navigate within their environment. Chemosensory neurons alert *C. elegans* to environmental chemicals, helping them find food and avoid danger (59). In this assay, chemoattractants are tested against control solutions to determine the effectiveness of a test solution, testing the chemotactic response of *C. elegans* seen in the chemotaxis assay. A control solution and test solution are micropipetted in four quadrants, each solution opposite each other, equidistant from the center of the plate, and from the *C. elegans*. The petri dishes are cooled to -20 ºC after inversion, then the *C. elegans* in each quadrant are counted after paralysis and calculated as a chemotaxis index (60). Figure 6 presents the equation for calculating the chemotaxis index.

The results highlight the ability of the test solution to attract *C. elegans* by stimulating their chemosensory neurons. In regards to testing polyphenols against amyloid-β inhibition, the behavioral phenotype from chemosensory neurons in which amyloid-β is expressed is tested (61). In an altered assay, worms are doused in a polyphenol, and used as the test in conjunction with a separate control

plate with unaltered *C. elegans*. The behavior of the worms treated with a test polyphenol is observed in comparison to the control plate. A proposed protocol utilizes benzaldehyde, a strong worm attractant in lower concentrations (65) to observe the change in neurological function of the *C. elegans*. If the polyphenol has anti-amyloidogenic properties, neurological function within the CL2355 strain is partially restored. In the assay, this would be seen as a greater ratio of worms moving twards benzaldehyde, the attractant, than seen in the control plate. The effectiveness with which the test solution stimulates olfactory neurons in the amyloid-β expressing strain CL2355 is quantified through the comparison of the chemotaxis index between test and control plates. In order to determine the effectiveness of a polyphenol, a statistical test is performed. A one-way Analysis of Variance (ANOVA) test is typically used. A p-value (obtained through ANOVA) < 0.05, is generally considered statistically significant. This indicates the polyphenol tested does have an effect in preventing amyloid-β toxicity in neurological functions in the *C. elegans* model (60).

Paralysis

The *C. elegans* model of Alzheimer's disease highlights age-dependent progressive paralysis, formation of amyloid deposits, and an increase of oxidative stress. The expression of the amyloid-β peptide in the body-wall muscle cells of several strains, including CL2006 and CL4176, produces a progressive paralysis phenotype, supporting the concept that "expression of human Aβ in *C. elegans* reliably recapitulates the abnormal structural phenotypes associated with toxic protein aggregates" (62). The CL4176 *C. elegans* transgenic strain has been engineered to have a temperature-sensitive mutation in its mRNA, such that that increasing the temperature to 25℃ triggers a severe and complete paralysis within 48 hours. Polyphenols are thought to reduce Aβ-induced paralysis in *C. elegans*. Thus, the utilization of this assay reveals the molecular mechanisms by which the polyphenols' act to mitigate paralysis (63). The addition of inhibitory agents to prevent amyloid aggregation changes the rate of paralysis in the worms, alters their response to neurotransmitters, and results in a decrease in chemotactic ability. The paralysis assays enable efficient visual monitoring of the various molecular processes leading to Aβ aggregation and demonstrate that a "decrease in Aβ mediated pathology in response to suppression of dld-1 supports the notion that decreased energy metabolism is neuroprotective" (64). The anti-amyloidogenic effects of the polyphenols are measured by spotting them on the worm plates and measuring their relative rates of paralysis. The protocol is performed on the standard nematode growth media (NGM) plates (65), with the tested polyphenol applied to the test plates prior to the assay. Gravid adult worms are transferred onto NGM plates spread with E.coli OP50 uracil auxotroph. A fraction of the second-day gravid adults are transferred to different NGM plates, which are then transferred to an incubator. The fraction of unparalyzed worms on each plate is converted into a percentage, and the average percentage is plotted against the time from temperature increase initiation to generate a paralysis curve (65).

Future Applications and Challenges

Despite a significant body of research indicating the protective effects of polyphenols, the magnitude of this protective effect is still ambiguious. Out of a group of 24 randomized, controlled human trials examining the

neuroprotective effects of dietary polyphenols examined in a review by Colizzi, 12 found a significant positive correlation with reduced cognitive decline, while 5 found no correlation, and 7 found mixed results. No conclusive results were obtained regarding the antioxidant protective effect of phenolic acid and flavonoid compounds (1). Extensive meta-analysis carried out by Lamport and Williams, found that the correlation between polyphenols and a negative effect on cognitive decline was tentative at best; at issue was the heterogeneity between the different studies and meta-analyses examined, so while general effects could be discerned, differences between specifics like efficacy of doses, duration of treatment, or sensitivity in populations or cognitive domains prevented statistical significance from being achieved (2).

Dietary polyphenol treatment analyses were also plagued with bioavailability issues. The most common example of this is curcumin, with a well documented history as a potential treatment for Alzheimer's, and strong arguments for its beneficial dietary effects. However, upon uptake into the body, curcumin is rapidly metabolized and converted to various less effective metabolites, such as curcumin sulfate or hexahydrocurcuminol. There are currently various efforts underway to remedy this issue, such as using adjuvants such as piperine to interfere with glucuronidation, implementing liposomal curcumin treatments, forming curcumin nanoparticles and administering those instead, using a curcumin-phospholipid complex as an alternative, or simply using a structural analogue of curcumin less vulnerable to metabolic degradation (3). A major factor in this strong metabolic activity against polyphenols is the generation of secondary metabolites by the gut biota to create glucuronidated, sulfated, and/or methylated products. Many red wine polyphenols, another target of therapeutic inquiry, face this issue; dosing with grape seed polyphenol extract resulted in significantly high plasma concentrations, but yielded insignificant results in terms of accumulation in the brain unless repeated high doses were administered. Repeated ingestion would be required, simply to maintain a significant plasma concentration of the grape seed polyphenol extract. Additionally, while initial evidence suggests that select polyphenols would be able to cross the blood-brain barrier (BBB), there is still an absence of further and specialized research on the matter. Resveratrol normally has poor BBB permeation, however, nanoencapsulation with lipid core nanoparticle significantly increased permeation (4). A useful tool to evaluate ADME before beginning clinical trials is the free web tool SwissADME, which evaluates drug candidates based on adherence to the Lipinski Rule of 5, predicted blood barrier permeation, drug-likeness, and various other pharmacokinetic parameters (5). Thus, while bioavailability is a significant roadblock in the implementation of polyphenols as anti-AD drugs, it can be remedied via computational analysis to select more favorable candidates, as well as preparing more effective formulations, including bioenhancers or nanoparticle encapsulation.

Additionally, there is room for improvement in terms of in vitro and in vivo testing of polyphenols. *C. elegans* is a common model organism used in the laboratory for antioxidant studies because it contains thousands of genes that are also found in the human genome (1). The organism is useful for studying diseases like Alzheimer's and Parkinson's because it serves as a good model for these diseases. *C. elegans* worms have short life cycles, lasting around two to three weeks and have several

tissues that perform the same functions as those in the human body (2). Because of the small size of their cells and organs and their relatively simple anatomy, it is difficult to test various tissue or organ specific drugs in vivo using these worms (3). Being that *C. elegans* are a completely different species, it is not fully accurate to translate the results obtained from in vivo experiments using *C. elegans* to humans. The observations from in vitro and in vivo experiments can be affected by various factors related to the *C. elegans* worms such as worm strain and conditions, and when treatments are translated into humans, the results can be altered by the patient's demographics, conditions, and other external factors (4) . Therefore, studying the effects of polyphenols on amyloid β aggregation in these worm strains cannot predict how the polyphenols can treat Alzheimer's in humans. Studying *C. elegans* does help determine which polyphenols show potential as an Alzheimer's treatment, as well as weeding out polyphenols that are not effective or can be harmful.

Furthermore, Alzheimer's disease (AD) is very difficult to diagnose due to its superposition with general age related cognitive decline, including the loss of memory (4). Memory loss, one of the most prominent symptoms of Alzheimer's, manifests differently for each person. In this sense, AD might present itself differently in each patient, therefore it is hard to identify. Unlike other common diseases like diabetes and heart disease, there are no biomarkers and no specific set of screenings or tests that a patient must go through in order to be definitively diagnosed. Although certain tests exist to check for biomarkers that are present in Alzheimer's patients, the connections are insignificant and therefore there is no guarantee that the patient actually has Alzheimer's. This is why it is very difficult to start clinical trials for Alzheimer's patients; there is no way to monitor the onset and progression of the disease. However, new studies are being developed to bridge this gap of uncertainty in diagnosing Alzheimer's patients. Liquid-liquid phase separation (LLPS) is a new in vitro method that studies the formation of tau droplets upon being placed in specific buffers that mimic physiological conditions linked to neurodegenerative diseases such as Alzheimer's. More specifically, LLPS causes "Phase separation into liquid droplet-like structures allows rapid, reversible condensation of specific proteins and nucleic acid molecules into discrete assemblies that dynamically exchange biomolecules with the surrounding cytoplasm and nucleoplasm (6)". Additionally, new research is being done that suggests how blood tests could be used to monitor the presence and progression of brain disease (2). Nancy Ip, a neuroscientist from Hong Kong University of Science and Technology has found a way to detect the progression of Alzheimer's disease using blood tests. The existence of a set of specific 19 blood proteins can indicate how extensively the disease has affected the brain (3). These specific biomarkers could be used to identify the stage of Alzheimer's the patient is experiencing, which allow the clinical scientists to maintain that all the trial participants are at the same stage of AD progression. Additionally, studies indicate that low oxidative levels of the brain can be a sign of early-onset Alzheimer's. Reactive oxygen species (ROS) are produced in excess in AD patients, which lead to "several harmful effects including DNA, lipid, and protein damage" (5). Thus, these patients also have elevated levels of naturally-produced antioxidants since they are trying to combat the excess of free radicals in the body (1). This causes an imbalance of antioxidants and oxidants in the body which

puts the patient into a state of oxidative stress. In sum, being able to detect these signs of oxidative stress could catch the disease early on and increase the chances of managing Alzheimer's symptoms.

Since some clinical trials specifically determine the antioxidant levels in the trial participants to identify their AD progression stage, it is important to have a baseline value of antioxidant status in order to be admitted into the trial. If patients regardless of their antioxidant status are admitted into the trial, then their different baselines may lead to incoherent results. Recent studies have found that because these trials do not take the antioxidant status into account as a baseline, "such indiscriminate enrolment could perhaps account for the negative results of antioxidant trials recently emphasized by a meta-analysis" (66). Additionally, because it has been suggested that the composition of an individual's microbiome affects antioxidant status, the dietary baseline of trial participants also should be taken into account as part of enrollment criteria into a clinical trial. The microbiome plays a key role in driving an individual's health, and thus their susceptibility to disease (67). Diet is the main contributor to maintaining a well-established microbiome, so taking into account; and controlling; an individual's diet prior to – and during - their enrollment in a trial is essential in maintaining a constant baseline. Furthermore, it has been shown that microbiome changes occur within weeks of a dietary change. This means that; unless normalized prior to enrollment and during the trial; the antioxidant and/or immunological status of a patient can change during the clinical trial thereby confounding the trial results.

Conclusion

Plant-derived natural product polyphenols may have the capability to inhibit the mutation of the amyloid-β protein, thus creating a treatment for Alzheimer's. More specifically, amyloid-β proteins have been connected to cell apoptosis, which may lead to diseases such as Alzheimer's. A possible treatment for such disorders could be to inhibit the amyloid-β proteins to prevent them from aggregating and interfering with cellular processes. Tau proteins play a role in the stability of neural microtubules that balance human neuronal function, hence mutations in these proteins may produce neurological disorders such as Alzheimer's disease. The function of these proteins depends upon the regulated process of phosphorylation; hyperphosphorylation causes the filaments to form tangles and malfunction, therefore creating an ineffective and detrimental protein (11-12). Three forms of prion disease stem from abnormalities in prion proteins. These diseases are most likely caused by the conversion of PrPC to PrPSc, which causes neurodegeneration and makes the prion proteins toxic to cells. Our project focuses on using polyphenols to inhibit the proteins from mutating. Polyphenols are the most diverse group of phytochemicals, possess one or more aromatic phenolic rings, and can be categorized into flavonoids, phenolic acids, stilbenes, or lignans. Polyphenols are typically used to protect against cytotoxic and oxidizing factors (3). Several assays test the effectiveness of these polyphenols. Thioflavin T is a benzothiazole dye that exhibits increased fluorescence when bound to amyloid fibrils, which is associated with the presence of cross-β structure (45). Congo Red is an azo dye that binds to amyloid-β proteins and can therefore be used to study these proteins in vitro. The Congo Red assay, combined with the

Thioflavin T assay, can be used to observe the structures and aggregation of amyloid-β proteins by determining changes in light absorption. The turbidity assay is another method to test the efficacy of polyphenols against amyloid-β. Turbidity measures the amount of light that passes through a given sample (53). The turbidity assay can measure the protein aggregate formed by the amyloid-β protein, determining the effectiveness of aggregate formation across the different polyphenols tested (54). An essential element of *C. elegans*' neuronal function is the ability to possess chemosensory neurons (59). Therefore, the chemotaxis assay can accurately measure the capability of the polyphenols test solutions to attract *C. elegans* (60). Lastly, the paralysis assay can be used to compare the different properties of polyphenols, in order to inhibit the amyloid-β protein. The *C. elegans* strain used contains a temperature-sensitive mutation, so that increasing the temperature can result in an absolute paralysis. The utilization of the paralysis assay allows visual monitoring of the various molecular processes, such as changes in the rate of paralysis, altered responses to neurotransmitters, and decreases in chemotactic ability, modulated by Aβ aggregation (63).

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Abbreviations

Aβ - Amyloid β
AD - Alzheimer's Disease
PrP^{C} - Cellular Prion Protein
PrP^{Sc}- Scrapie Isoform Prion Protein
NMR - Nuclear Magnetic Resonance
TSE - Transmissible Spongiform Encephalopathies
EGCG - Epigallocatechin Gallate
ROS - Reactive Oxygen Species
RNS - Reactive Nitrogen Species
SOD - Superoxide Dismutase
ELISA - Enzyme-linked Immunosorbent Assay
APP - Amyloid Precursor Protein
PBS - Phosphate Buffer Solution

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