Molecular interactions with redox sites and salt bridges modulate the anti-aggregatory effect of flavonoid, tannin and cardenolide moieties against amyloid-beta (1–42) in silico

Rafael Vincent M. Manalo¹

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Abstract In this study, the interactions of flavonoid, tannin and cardenolide moieties as well as their known metabolites were docked against the apolar NMR structure of the aggregatory amyloid-beta fragment ($A\beta_{1-42}$). Results showed that the catechin moiety favorably bound $A\beta_{1-42}$ peptide at Asp23, Asn27, Ser26 and Glu22 residues, with chalcone similarly binding the middle region of the peptide. Remarkably, hippuric and ferulic acids exhibited hydrophobic interactions with $A\beta_{1-42}$ at the latter portion of the peptide, possibly blocking the salt bridges formed by Glu22-Lys28 which stabilizes Phe19-Gly25, as well as the β -sheet Leu34-Gly38 that are known to exist in peptide aggregation. Meanwhile, the metabolites of hydrolyzable tannins, such as urolithin A and gallic acid, exhibited H-bonding interactions with different residues of $A\beta_{1-42}$, including Asp1, Asp23 and hydrophobic interactions by gallic acid planar ring to the Hsd6 residue. The coverage was lessened in pyrogallol, suggesting that gallic acid loses its efficacy when further metabolized. Lastly, the different binding poses of the cardenolide moiety interacted with Hsp6 (protonated His) and Tyr10 via hydrophobic interactions. Due to these interactions, the large polycyclic moiety of the ligand would also block further interactions with Hsd6 (prototropic tautomer of His), Asp7, Ser8 and Gly9 that are integral to His6-His13-His14, Arg5-Asp7 and Leu34-Gly38 β -sheets, salt bridges in Glu22-Lys28 and turn conformation Phe19-Gly25. Together, these data suggest that the known metabolites of anthocyanins and hydrolyzable tannins contribute the most effective

anti-aggregatory interactions with $A\beta_{1-42}$, with an unexpected role for cardiac glycosides such as the cardenolide moiety. These bring to light the important role of metabolism in vivo, and suggests further investigation on the effects of these metabolites when concentrated in vivo.

Keywords Amyloid-beta aggregation · Single molecule docking · Anti-aggregatory compounds · Phytochemical screening

Introduction

Recent studies have shown the promising effects of phytochemicals in preventing the aggregation of proteins that often perturb homeostasis, induce stress, and trigger cell survival or cell death mechanisms. For instance, flavonoids such as anthocyanosides and some flavones were shown to be anti-aggregatory (Zaragoza et al. 1985; Bojic et al. 2011; Villaseñor 2011). Further, the anti-amyloidogenic and anti-aggregatory properties of tannic acid has been observed (Ono et al. 2004; Lee et al. 2009). These studies, along with others showing the potential of naturally-derived compounds, provide an alternative turn from conventional criteria in drug discovery for proteinopathies such as Alzheimer's disease (AD). One among many of the criteria for AD drug discovery includes antioxidant activity (Mecocci and Polidori 2012; Feng and Wang 2012; Galasko et al. 2013), which is based on associations that protein oxidation can lead to aggregation (Mirzaei and Regnier 2008) and eventual deposition such as amyloid- β ($A\beta$) deposits observed in the brain of patients with AD. Further, recent studies have shown the role of metals as cofactors that associate with $A\beta$ and catalyze the formation of reactive oxygen species (Maynard et al. 2005; Ott et al. 2015), all of which

✉ Rafael Vincent M. Manalo
rmmanalo3@up.edu.ph

¹ Department of Biochemistry and Molecular Biology,
College of Medicine, University of the Philippines Manila,
Ermita, 1000 Manila, Philippines

maintain the use of antioxidants as promising treatment for A β -induced pathologies in AD. However, prevention of ROS is a rather simplistic approach to neuroprotection. When proteins unfold, the endoplasmic reticulum (ER) senses the resulting homeostatic perturbations and in turn activate the unfolded protein response (UPR), which upregulates chaperones to assist proper protein folding and proteins that direct these persistently unfolded proteins for degradation to the

proteasome (Schröder and Kaufman 2005). However, in the presence of persistent stress—such as persistent amyloidogenesis and aggregation in AD—the ER tips the balance between cell survival and cell death signals and activates pathways directed to apoptosis (Manalo and Medina 2017). This pathway, which may be independent on antioxidant activity, reveal the aggregatory phenomenon of A β fragments as a potential and more promising therapeutic target

Fig. 1 Flavonoids and their metabolites interact favorably with $A\beta_{1-42}$. **a** Catechin interacts with $A\beta_{1-42}$ at residues Asp23, Ser26 and Asn27 with a Fullfitness value of $-38,469.625$ kcal/mol, where (in the peptide) green = Asp, blue = Asn, and red = Ser. **b** Five representative interactions of catechin showcasing preference for Glu22, Asp23, Ser26 and Asn27. The binding mode energies range from $-38,469.62$ to $-37,972.68$ kcal/mol, and are colored in green. All possible interactions are clustered in the middle region. **c** Hippuric acid interacts with $A\beta_{1-42}$ at Lys16 with a Fullfitness value of $-37,815.79$ kcal/mol, where (in the peptide) red–orange = Lys. **d** Five representative interactions of hippuric acid showcasing mainly hydrophobic interactions with the latter half of the peptide. The binding mode energies range from $-37,815.79$ to $-37,557.74$ kcal/mol, and are colored in green. **e** Ferulic acid interacts with $A\beta_{1-42}$ at Lys16 with a Fullfitness value of $-37,851.61$ kcal/mol, where (in the peptide) red–orange = Lys. **f** Five representative interactions of ferulic acid with $A\beta$ peptide showcasing mainly hydrophobic interactions spanning the body of the peptide with H-bonds at Asp1. The binding mode energies range from $-37,851.61$ to $-37,641.086$ kcal/mol, and are colored in green. **g** Chalcone interacts with $A\beta_{1-42}$ between the R-groups of Ser26 and Asn27 with a Fullfitness value of $-38,098.48$ kcal/mol, where (in the peptide) red = Ser and blue = Asn. **h** Five representative interactions of chalcone with $A\beta$ peptide showcasing mainly hydrophobic interactions between Asp7 and Glu11, and between Phe19 and Ile32. The binding mode energies range from $-37,851.61$ to $-37,641.086$ kcal/mol, and are colored in green. Ligands were mined from PubChem and Chempidder

for AD. Recently, Manalo et al. showed that coconut leaf extract (CLE) reduced $A\beta_{1-42}$ aggregation and induced-paralysis in the muscle cells of *C. elegans* independently of antioxidant activity (2017), exemplifying the potential of non-antioxidant anti-aggregatory phytochemical compounds in treating AD. However, many of the phytochemicals tested for anti-aggregatory properties have so far been discounted of metabolic participation. To date, there is scarce data showing anti-aggregatory effects of phytochemical metabolites, which in spite of being biologically relevant has not been focused on. These motivated the study presented herein. To the best of my knowledge, this is the first study that establishes an in silico basis of anti-aggregatory properties of anthocyanins, tannins and cardiac glycosides, with greater emphasis on their known metabolites that are rather convenient since these metabolites are always derived from the phytochemicals mentioned, regardless of functional group novelty.

Materials and methods

The elucidated protein structure of $A\beta_{1-42}$ was obtained from the nuclear magnetic resonance (NMR) solution structure of Crescenzi et al. via *RCSB PDB*, under the criteria of being the most thermodynamically favorable structure in terms of obeying physical constraints (Crescenzi et al. 2002). The base molecular structures (no functional groups) of anthocyanins, hydrolyzable tannins, cardenolides and their known metabolites in vivo were mined from Pubchem and

ChemSpider, and were then docked against the $A\beta_{1-42}$ peptide using *Swissdock* (Grosdidier et al. 2011a; Grosdidier et al. 2011b). Binding modes were scored according to their average Fullfitness per element (Grosdidier et al. 2007), and were visualized using *JSmol* and *UCSF Chimera* (Pettersen et al. 2004). Interactions between the ligand molecules and the target protein (in this case, $A\beta_{1-42}$), were emphasized via color-coding. To ease the process, color-coding was done by amino acid type. Hydrogen bonds were emphasized in green lines from the molecule to the protein. To assess affected amino acid residues, residue deletion was performed, which would result in the loss of hydrogen bonding emphasis. This confirms H-bonding interactions between the molecule and the suspected residues. For non H-bonding interactions, the residue number and amino acid type were determined on the affected portions of $A\beta_{1-42}$. In this paper, only the top 5 interactions with the most thermodynamically favourable binding modes were assessed. All assessments were done in *UCSF Chimera*.

Results

Previous phytochemical analysis showed the presence of flavonoids, tannins and cardenolides in CLE that reduced $A\beta_{1-42}$ in transgenic *C. elegans* (Manalo et al. 2017). This motivated the study of molecular interactions of the base moieties of these compounds and their known metabolites in vivo to rationalize in silico the overall anti-aggregatory effect of the extract used. For flavonoids, the structure of catechin was used as a representative molecule, since catechins are secondary metabolites usually found in the vascular portion of plants (Fig. 1a, b).

Remarkably, catechin bound the $A\beta$ protein at the most favorable binding mode (Fullfitness: $-38,469.62$ kcal/mol) to three residues, namely Asp23, Asn27 and Ser26, and to Glu22 albeit at a lower yet favorable docking score. Further, the estimated ΔG s ranged from -25.97 to -63.47 kcal/mol in different conformations, suggesting spontaneous arrangements to the computed ligand poses as it interacts with the protein.

The possible direct toxicity of $A\beta_{1-42}$ as suggested by its similarity to the viral fusion domain, at least in this structure (Crescenzi et al. 2002), implies that direct interaction with the peptide may be protective of AD pathology. Further, all conformations of catechin seem to favor interactions with the middle region of the $A\beta$ peptide (Fig. 1b), particularly bound between amino acids 22 to 27— suggesting a predictable interaction between $A\beta$ and catechin. Known intestinal and microfloral metabolites of anthocyanins in vivo in humans were also docked, including chalcone and the major phenolic metabolites hippuric and ferulic acid (Czank et al. 2013; Kalt et al. 2014; de Ferrars et al. 2014).

Apparently, hippuric and ferulic acid that are major phenolic metabolites of anthocyanin consumption interacted with A β at Lys16, with a minimum Fullfitness of $-37,815.79$ and $-37,851.61$ kcal/mol and ΔG of -20.08 and -21.14 kcal/mol respectively, suggestive of spontaneous conformation that is also thermodynamically favorable for ligand–protein interactions. Further, both exhibited hydrophobic interaction with A β_{1-42} – the former mainly at the latter half of the peptide through its benzene ring, and the latter spanning the body of the peptide in much the same manner (Fig. 1c–f). Through these interactions, the salt bridges formed by Glu22–Lys28 that stabilizes Phe19–Gly25, as well as the β -sheet Leu34–Gly38 that are known to exist in peptide aggregation, might not form properly. This would also include known β -sheets His6–His13–His14 and Arg5–Asp7 (Shi et al. 2016; Boopathi and Kolandaivel 2016). Interestingly, the most favorable binding pose of chalcone was between Ser26 and Asn27, which are both polar amino acids. This may be due to the stability conferred to that region brought by the moving of electronegative oxygens of Ser and Asn away from each other, thereby lessening electron repulsion (Fig. 1g). This conformation has a Fullfitness of $-38,098.48$ kcal/mol and a ΔG of -33.15 kcal/mol—supportive of such a hypothesis.

Similarly, possible binding modes of chalcone were localized in the same middle region as in catechin, with an additional localization between residues 7 to 11 (Fig. 1h). Thus, it is expected that chalcone would act in the same manner as catechin, provided that the microenvironment is the same.

Due to the presence of hydrolyzable tannins in the CLE, it was postulated that metabolites of such tannins could also contribute to the efficacy of the leaf extract. We previously hypothesized the possible roles of urolithin A, gallic acid and pyrogallol in modulating the overall anti-aggregatory effect of CLE (Manalo et al. 2017). Hence, their moieties were also screened using *Swissdock* and visualized via *UCSF Chimera*. Urolithin A interacted with A β as well, particularly with the front via hydrophobic interactions with its polycyclic moiety, and with the middle region via H-bonding to Asp23, Ser26, and Gly29 (Fig. 2a). Notably, gallic acid exhibited greater use of its electronegative oxygen for H-bonding with various residues, including Asp1, Gly9 and Asp23. Further, it showed a combination of H-bonding and hydrophobic interaction, as exemplified by its bond with Asp1 and interaction with Hsp6 (blue) via its planar phenolic ring (Fig. 2c, d). Similar with urolithin A, gallic acid covered the front and middle regions of the peptide, with the most favorable binding mode interacting via H-bonds to Lys16 and Asp23.

Similarly, pyrogallol interacted with familiar residues that gallic acid had interacted with, albeit with less coverage than the former, and with more H-bonds (Fig. 2e, f). This implies a lesser desired effect as gallic acid is metabolized

in vivo. The same was done for the cardiac glycosides, with the cardenolide moiety as a representative ligand (Kolenda et al. 1971; Rietbrock et al. 1977). In contrast, the most favorable binding pose (Fullfitness: $-38,028.785$ kcal/mol) of the cardenolide moiety interacted with A β_{1-42} in the latter part of the first ten residues, particularly with Hsp6 (protonated His) and Tyr10 via hydrophobic interactions (Fig. 3a). Due to these interactions, the large polycyclic moiety of the ligand also blocked further interactions with Hsd6 (prototropic tautomer of His), Asp7, Ser8 and Gly9. This is noteworthy, since β -sheet conformations in A β aggregation involve His6–His13–His14, Arg5–Asp7, Asp1 and Ser8 (Shi et al. 2016; Du et al. 2011). To this end, the most plausible binding mode could block salt bridges at these residues, with a ΔG of -27.739 kcal/mol suggesting that such a conformation could happen spontaneously. Whereas catechin binds preferably to one peptide region only, cardenolide interacts, mostly via hydrophobic interactions, with the front, middle and end portions of A β_{1-42} (Fig. 3b). Specifically, the different binding poses of the cardenolide moiety could block sheet conformations such as His6–His13–His14, Arg5–Asp7, Leu34–Gly38 (Shi et al. 2016), salt bridges in Glu22–Lys28 and turn conformation Phe19–Gly25, which are known to occur in A β aggregation (Boopathi and Kolandaivel 2016). Further, the N-terminal and C-terminal residues of the A β_{42} peptide interacted favorably with the cardenolide moiety, suggesting that the suspected role of the N-terminal in ROS production may be inhibited by loss of availability for Cu²⁺ ions (Tiiman et al. 2016). However, the increased hydrophobicity in this region may promote fibrillation, just as how N-terminal lipid conjugation could accelerate fibrillation kinetics of A β (Adler et al. 2017), which may explain the inability to completely prevent A β aggregation. More importantly, the chances that the cardenolide moiety would interact with the peptide, the presence of a glucoside moiety in plasma, the peptide residues exposed and macromolecular crowding all likely contribute further to possible variations in vivo.

Discussion

Molecular docking of flavonoids, hydrolyzable tannins, cardenolide and their metabolites in silico seem to support some of their experimental anti-amyloidogenic and anti-aggregatory effects. Some metabolites, including ferulic and hippuric acid, urolithin A, gallic acid and cardenolide, interact with either the C- or N-terminal of the peptide, which are hypothesized to cause oxidation of metal ions, primarily Cu²⁺. By blocking the N-terminal, for instance, metal oxidation would not occur, which would explain why the effect of CLE in *C. elegans* was not at par with its scavenging effect in vitro; firstly, because the phytochemicals

Fig. 2 Metabolites of hydrolyzable tannins interact favorably with A β ₁₋₄₂. **a** Urolithin interacts with A β ₁₋₄₂ at residues Asp23 with a Fullfitness value of $-38,202.45$ kcal/mol, where (in the peptide) red-orange = Asp. **b** Five representative interactions of urolithin A showcasing preference for Asp23, Ser26 and Gly29. The binding mode energies range from $-38,202.65$ to $-37,838.17$ kcal/mol, and are colored in green. All possible interactions are clustered in the middle and front regions. **c** Gallic acid interacts with A β ₁₋₄₂ at Lys16 and Asp23 with a Fullfitness value of $-37,903.57$ kcal/mol, where (in the peptide) purple = Lys and red-orange = Asp. **d** Five representative interactions of gallic acid showcasing H-bonding and hydropho-

bic interactions with the front and middle regions of the peptide. The binding mode energies range from $-37,903.57$ to $-37,589.89$ kcal/mol, and are colored in green. **e** Pyrogallol acid interacts with A β ₁₋₄₂ at Asp23 with a Fullfitness value of $-38,079.89$ kcal/mol, where (in the peptide) red-orange = Asp. **f** Five representative interactions of pyrogallol with A β peptide showcasing mainly hydrophobic interactions spanning the body of the peptide with H-bonds at Phe4, Asp23, Ser26, Gly29 and Ile32. The binding mode energies range from $-38,079.89$ to $-37,792.93$ kcal/mol, and are colored in green. Ligands were mined from PubChem

were already metabolized; secondly, because the metabolites instead prevent redox reaction to occur by blocking redox sites, which is an indirect approach and is expected to have a different trend than the scavenging effect of CLE *in vitro*. Further, by interacting with the A β peptide in regions where oligomerization and salt bridges are expected to form, these metabolites may indeed prevent further aggregation.

In the docking of flavonoids, particularly catechin and known anthocyanin metabolites chalcone, hippuric and

ferulic acid, direct anti-aggregatory interactions with the middle portion of the peptide were observed for both catechin and chalcone. These suggest that the effect of these compounds, if any, would be similar in a given microenvironment. Further, a larger coverage was found when anthocyanin metabolites were docked, such as residues involved in salt-bridge and β sheet formation integral to the aggregation of A β ₁₋₄₂. Once bound, hippuric and ferulic acids would be able to block formation of these secondary protein

Fig. 3 Cardenolide moiety interacts favorably with A β_{1-42} . **a** Cardenolide interacts with A β_{1-42} at residues Hsp6 and Tyr10 with a Fullfitness value of $-38,028.785$ kcal/mol, where (in the peptide) green = Hsd, blue = Hsp, Red = Asp, orange = Tyr, yellow = Gly, purple = Ser. **b** Six representative interactions of cardenolide with A β

peptide showcasing a wide range of preferred interactions. The binding mode energies range from $(-38,028.72)$ to $(-37,661.44)$ kcal/mol, and are colored in green. All possible interactions are spread around the peptide. Ligands were mined from PubChem

structures, eventually leading to the loss of efficient A β aggregation. In general, these results imply that anthocyanins may as well be more effective after it is metabolized, with promising anti-amyloidogenic and indirectly antioxidant (by preventing Cu^{2+} His complex formation) properties.

For hydrolyzable tannins, metabolites were docked—mainly because of the hydrolyzable nature of the phytochemical that makes it prone to degradation upon digestion in vivo. Its well-known metabolite, urolithin A, exemplified direct interactions with the middle region of the A β_{1-42} fragment. This possibility to block oligomerization at the front and middle region (23–29) of the peptide, combined with its known function to extend *C. elegans* lifespan and improve muscle strength, may indeed be suggestive of its promising role in the treatment of muscular proteinopathies. Further studies on the applications of urolithin A is highly suggested. This may as well explain previous findings on why the lowest concentration of CLE was found to be the most effective in preventing A β -induced paralysis in CL4176 [23], which then decreased in effect probably due to cytotoxicity by other present phytochemicals.

Lastly, cardenolides showed promising interactions with Hsd6 (prototropic tautomer of His), Asp7, Ser8 and Gly9. Since the formation of β sheets integral for aggregation involves these residues, it is noteworthy that cardenolides can most likely prevent aggregation by blocking β sheet formation. It was noted that cardiac glycosides are potentially bioavailable for AD treatment, since they may accumulate in the central nervous system (Smith 1985), and this warrants investigation on the alternative yet promising effects of cardiac glycosides on A β_{1-42} aggregation.

Admittedly, this approach has certain limitations. For one, the structures docked against the A β_{1-42} peptide were moieties universally-conserved among the phytochemicals. Whereas docking of metabolites provides a better estimate

of efficacy in vivo, moieties do not account for differing functional groups that are expected to vary in every plant extract. Further, the interactions between the moieties and A β_{1-42} were simplistic, and does not account for macromolecular crowding and quinary interactions that usually occur inside the cell. Nonetheless, the docking of metabolites provided a better estimate, since these metabolites will always be present when the phytochemicals are digested in vivo, regardless of functional group. Further studies exemplifying microenvironment interactions are suggested to confirm the interactions in silico. Lastly, *Swissdock* returns docking information based only the thermodynamic spontaneity and favorable interactions by which molecular structures bind to the peptide. Therefore, there are no apparent information on the affinity of binding, and further studies on binding affinities are suggested to improve the estimation of A β_{1-42} interactions.

Conclusions

This study is the first to present interactions between the A β_{1-42} peptide fragment and moieties of flavonoids and cardenolides, and their metabolites chalcone, hippuric and ferulic acids, as well as the known metabolites tannins urolithin A, gallic acid and pyrogallol, which have also shown therapeutic effects in recent years. Their interactions with A β_{1-42} spontaneously blocked redox sites and sites for β -sheet and salt bridge formation, indicating their role in being anti-aggregatory compounds in silico. This result sheds some light on unconventional criteria for AD drug discovery, which more often than not focuses on antioxidant or acetylcholinesterase (AChE) inhibitory activities. Protein folding is of relevance in the cell, as persistent folding can activate stress response that may

lead to neurodegeneration, in the context of AD. The best interactions were found for hippuric and ferulic acid, as well as with the cardenolide moiety. For the former, this suggests that metabolism of phytochemicals has a major role in determining their efficacy *in vivo*. For the latter, an unexpected anti-aggregatory pleiotropic effect was found for cardiac glycosides, suggesting further studies on this regard.

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Compliance with ethical standards

Conflict of interest The author discloses no conflict of interest.

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