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Myricetin: A Naturally Occurring Regulator of Metal-Induced Amyloid-β Aggregation and Neurotoxicity

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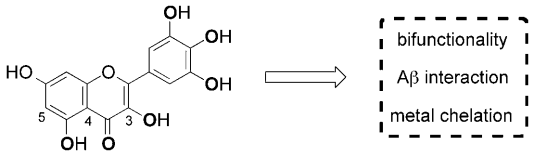
One of the most severe and incurable forms of neurodegeneration, Alzheimer's disease (AD), is characterized in the brain by the accumulation of aggregated amyloid-β (Aβ) peptides.[1–3] In the diseased brain, elevated concentrations of metals, such as Fe, Cu, and Zn, are found in Aβ plaques.[1–4] It has been proposed that metal ions, such as Cu^{II} and Zn^{II}, can bind to Aβ; this causes enhanced peptide aggregation, and in the case of redox active metal ions (e.g., Cu), the generation of reactive oxygen species (ROS) leading to oxidative stress and neuronal death.[1–9] While peptide aggregation and oxidative stress have been implicated in AD progression, the role of metal ions associated with Aβ species in the development of this disease remains unclear.

To clarify the function of metal ions in Aβ-related pathological events, small molecule-based tools that contain bifunctionality for probing both metal ions and Aβ have been sought.[10–14] Several small molecules have been fashioned according to a rational structure-based design strategy to target metal-associated Aβ species (metal–Aβ species) and to interrogate metal-induced Aβ aggregation and neurotoxicity.[3,9–14] Due to the range of possible conformations of metal–Aβ that could be involved in AD neuropathogenesis,[2–4,7] discovery of novel structural frameworks that can target these species might advance progress for this design strategy. One tactic to identify new classes of basic structural scaffolds is through screening of naturally occurring compounds, such as flavonoids.

Flavonoids are a class of polyphenolic compounds that are abundant in natural products, such as berries, fruits, and vegetables, and have been investigated as potential therapeutic agents in human diseases including cancer, cardiovascular disease, and AD.[15–22] These naturally occurring compounds have been shown independently to chelate metal ions and to interact with Aβ, suggesting their potential bifunctionality toward metal–Aβ species.[19–25]

One of the flavonoid compounds, myricetin (Scheme 1), previously demonstrated an anti-amyloidogenic effect through its reversible binding to fibrillar Aβ but not to the monomeric species.[21] In the case of its metal binding property, prior studies have shown that myricetin has multiple potential sites for metal chelation including positions between the 4-oxo and the 3- or 5-OH groups (Scheme 1) that can form complexes with a binding stoichiometry of 1:1 or 1:2, metal/myricetin.[24,25] Despite the known interactions of myricetin or other members of the flavonoid family with metal ions and Aβ, their influence on metal-induced Aβ aggregation pathways and neurotoxicity has not been investigated. Herein, we report that myricetin, exhibiting bifunctionality (metal chelation and Aβ interaction), was capable of modulating Cu^{II}- and Zn^{II}-induced Aβ aggregation and neurotoxicity in vitro and in human neuroblastoma cells. To the best of our knowledge, this is the first example to establish the reactivity of the flavonoid myricetin toward metal–Aβ species.



Scheme 1. Chemical structure of myricetin (3,5,7-trihydroxy-2-(3,4,5-trihydroxyphenyl)-4*H*-1-benzopyran-4-one). Potential donor atoms for metal chelation are highlighted in bold.

Before conducting reactivity studies of myricetin with metal–Aβ species, its metal binding properties were confirmed by UV/Vis experiments. In agreement with the previous reports, a bathochromic shift of the optical band was observed upon addition of one equivalent of $CuCl_2$ to myricetin (25 μM) in HEPES (20 mM), pH 7.4, NaCl (150 mM; ca. 377 to 450 nm, Figure S1 in the Supporting Information).[24] Similarly, a bathochromic shift in the optical spectrum (ca. 377 to 400 nm, Figure S1 in the Supporting Information) was visible when one equivalent of Zn^{II} was introduced to myricetin. Moving forward, to determine if metal chelation by myricetin was possible in the presence of Aβ, one equivalent of myricetin was added to a preincubated solution of Aβ (25 μM) and $CuCl_2$ or $ZnCl_2$ (25 μM). New absorption bands that resembled metal-incubated myricetin in Aβ-free solutions appeared (Figure S1 in the Supporting Information). These results suggest that myricetin could compete with Aβ for binding to metal ions, with possible implications for reactivity in metal-induced Aβ events (vide infra).

The reactivity of myricetin toward in vitro metal-induced Aβ aggregation was probed according to previously reported procedures (Figures 1 and 2).[12–14,26] Generally, if a small molecule can inhibit formation of or promote the disassembly of Aβ aggregates, more soluble, smaller-sized Aβ species will be produced. The relative M_W distribution of these Aβ species can be

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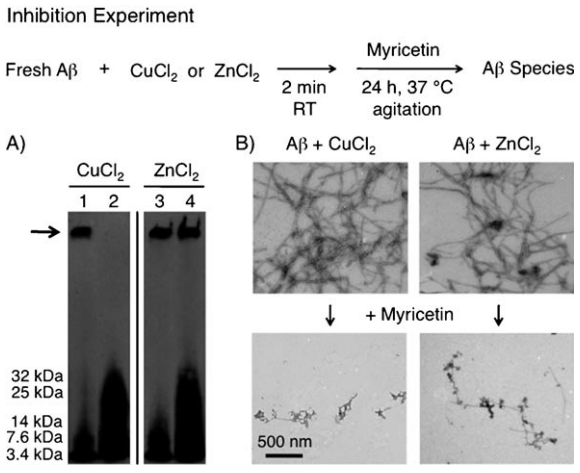

Figure 1. Inhibitory influence of myricetin on the formation of metal-induced Aβ aggregates. Top: scheme of the inhibition experiment. Bottom: A) Aβ species visualized by native gel electrophoresis by Western blotting with the anti-Aβ antibody, 6E10. Lanes: 1) Aβ + $CuCl_2$; 2) Aβ + $CuCl_2$ + myricetin; 3) Aβ + $ZnCl_2$; 4) Aβ + $ZnCl_2$ + myricetin. The black arrow denotes the position of the sample well at the gel entrance. B) TEM images of the samples from (A). Experimental conditions: [Aβ] = 25 μM, [$CuCl_2$ or $ZnCl_2$] = 25 μM, [myricetin] = 50 μM, pH 7.4, 37 °C, 24 h, constant agitation.

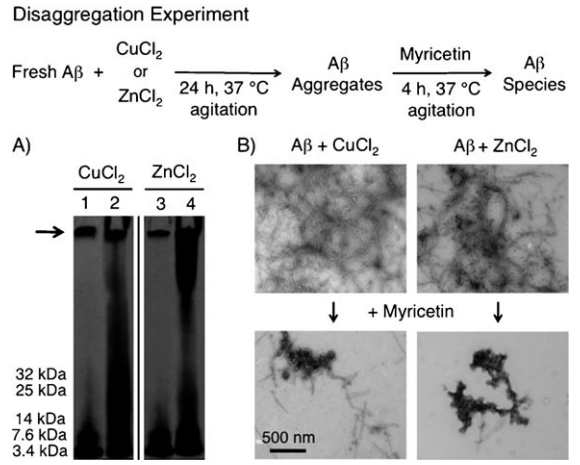

Figure 2. Transformation of metal-induced Aβ aggregates by myricetin. Top: scheme of the disaggregation experiment. Bottom: A) Aβ species visualized by native gel electrophoresis by Western blotting with the anti-Aβ antibody, 6E10. Lanes: 1) Aβ + $CuCl_2$; 2) Aβ + $CuCl_2$ + myricetin; 3) Aβ + $ZnCl_2$; 4) Aβ + $ZnCl_2$ + myricetin. The black arrow denotes the position of the sample well at the gel entrance. B) TEM images of the samples from (A). Experimental conditions: [Aβ] = 25 μM, [$CuCl_2$ or $ZnCl_2$] = 25 μM, [myricetin] = 50 μM, pH 7.4, 37 °C, 4 h, constant agitation.

illustrated by native gel electrophoresis with Western blotting because the smaller-sized peptide aggregates can penetrate and be separated on the gel matrix. On the other hand, large Aβ aggregates (i.e., mature fibrils) can be restricted at the entrance of the gel where the sample is loaded.[27,28] Furthermore, if preformed Aβ aggregates are disassembled by a compound, a wide M_W range of Aβ species can be generated. In the native gel, the amount of smearing from top to bottom (high to low M_W) can be an indication of the utility of the molecule for disaggregation. In conjunction with the native gel analysis, transmission electron microscopy (TEM) can be used to visualize morphological features of the generated Aβ species. Under our experimental settings that employ these methods together, monitoring the degree of Aβ aggregation (i.e., changes in M_W distribution of Aβ species) and/or morphologies of the Aβ aggregates can reveal how effectively a small molecule regulates Aβ aggregation and/or disaggregation in the presence or absence of metal ions.

To first understand if myricetin is able to prevent the formation of metal-induced Aβ aggregates (inhibition experiment, Figure 1),[12–14,26] fresh Aβ (25 μM) was treated with $CuCl_2$ or $ZnCl_2$ (25 μM) for 2 min followed by 24 h incubation with myricetin (50 μM). Myricetin distinctly inhibited the generation of Cu^{II}- and Zn^{II}-involved Aβ aggregates, and an increased amount of lower M_W Aβ species ($M_W \leq 32$ kDa) was found compared to myricetin-free metal–Aβ samples, as determined by native gel electrophoresis followed by Western blotting with anti-Aβ antibody, 6E10 (Figure 1). The addition of myricetin to the sample containing Aβ and Cu^{II} or Zn^{II} provided lower M_W Aβ species that could enter and be separated on the native gel. In contrast to the myricetin-untreated Cu^{II}– or Zn^{II}–Aβ samples, these Aβ species had conformations that were much less ordered, which were visualized by TEM (Figure 1 B). Taken together, these results demonstrate the ability of myricetin to modulate the production of Cu^{II}- and Zn^{II}-triggered Aβ aggregates.

According to the results in Figure 1, the extent of inhibition and resulting morphologies were dependent upon the metal ion present. Noticeably, no band was observed at the gel entrance in the Cu^{II}–Aβ sample with myricetin; only an increased amount of Aβ species with $M_W \leq 32$ kDa (compared to the myricetin-untreated Cu^{II}–Aβ sample) was detected. Unlike the Cu^{II}-induced case, the top band was present for the myricetin-treated Zn^{II}–Aβ sample along with an increase of lower M_W Aβ species, which implies that a mixture of Aβ aggregates existed. These differences in the Western blots correlated to the TEM results; only unstructured aggregates were shown for the Cu^{II} sample, whereas in the Zn^{II}–Aβ samples, a mixture of fibrillar and unstructured Aβ aggregates was visualized (Figure 1 B). Thus, both the Western blot and TEM results suggest that myricetin was able to control the formation of both Cu^{II}- and Zn^{II}-induced Aβ aggregates with greater reactivity observed for the Cu^{II}-related pathway (Figure 1).

In addition to the inhibition experiments, the ability of myricetin (50 μM) to disassemble metal-associated Aβ aggregates, which were generated by 24 h incubation of fresh Aβ (25 μM) with metal chloride salts (25 μM), was evaluated (disaggregation experiment, Figure 2).[12–14,26] Following only 4 h treatment, myricetin could fragment Aβ aggregates formed in the presence of Cu^{II} or Zn^{II} to afford a mixture of smaller-sized Aβ species that could be separated by the native gel, and which had assorted morphologies that were clearly distinguishable from the myricetin-free metal–Aβ samples by TEM (Figure 2). Similar to the results of the inhibition experiments, myricetin exerted different effects on the disaggregation of the Aβ species formed in the presence of either Cu^{II} or Zn^{II}. For the Cu^{II}-treated samples, a more dispersed M_W distribution of Aβ species

was indicated as visualized by the smearing in the native gel, particularly at about $\leq$32 kDa (Figure 2A). On the other hand, the myricetin-treated Zn^{II}–Aβ species were more concentrated around the low and high M_W areas with less smearing in the middle of the gel. A variety of morphologies (fibrillar and amorphous) were represented by TEM for both Cu^{II}- and Zn^{II}-treated Aβ species upon addition of myricetin, and were smaller and less ordered overall as compared to the myricetin-free Cu^{II}– or Zn^{II}–Aβ samples (Figure 2B). Overall, myricetin was effective at breaking down large, structured Cu^{II}– and Zn^{II}–Aβ aggregates, and additionally led to noticeable alteration of the M_W distributions and conformation of Aβ species.

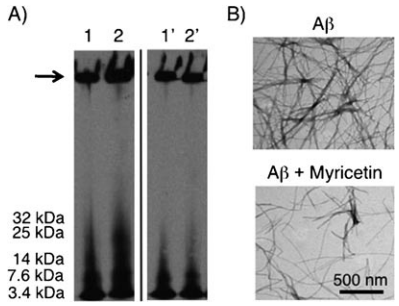

Figure 3. Metal-free inhibition and disaggregation studies using myricetin. A) Metal-free Aβ species visualized by native gel electrophoresis by Western blotting with the anti-Aβ antibody, 6E10. Samples from the inhibition experiments are presented in lanes 1 (Aβ) and 2 (Aβ + myricetin), and the disaggregation experiments are shown in lanes 1′ (Aβ) and 2′ (Aβ + myricetin). The black arrow denotes the sample well at the gel entrance. B) TEM images of metal-free Aβ (top) and metal-free Aβ + myricetin (bottom) from the inhibition experiment. Experimental conditions: [Aβ] = 25 μM, [myricetin] = 50 μM, pH 7.4, 37 °C, constant agitation.

For comparison of reactivity of myricetin with metal–Aβ species, metal-free inhibition and disaggregation experiments were conducted (Figure 3). In the absence of metal ions under the same conditions, partial inhibition of Aβ aggregation by myricetin occurred based on the slightly increased amount of Aβ species with $M_W \leq 32$ kDa; no discernable effect was presented in the disaggregation experiment (Figure 3A). By TEM, Aβ in the absence of myricetin was aggregated as long fibrillar structures (Figure 3). After treatment with myricetin, the generated Aβ aggregates had similar morphology to the myricetin-untreated metal-free Aβ species. These observations showed that myricetin was not able to significantly control metal-free Aβ aggregation, suggesting preferential reactivity toward metal-induced Aβ aggregation pathways.

Moving forward, we examined the ability of myricetin to attenuate neurotoxicity from metal–Aβ species in human neuroblastoma SK-N-BE(2)-M17 cells (M17) that were maintained in media containing fetal bovine serum (FBS, 10%; Figure 4).[12,13] The cytotoxicity of the metal ions and myricetin was measured by incubating the cells with a metal ion alone (10 μM), myricetin alone (20 μM), or myricetin and $CuCl_2$ or $ZnCl_2$ for 24 h. Under these conditions, Cu^{II}, Zn^{II}, myricetin, Cu^{II} with myricetin, and Zn^{II} with myricetin were not toxic after 24 h incubation relative to the viability of cells containing DMSO (1%; *v/v*), as

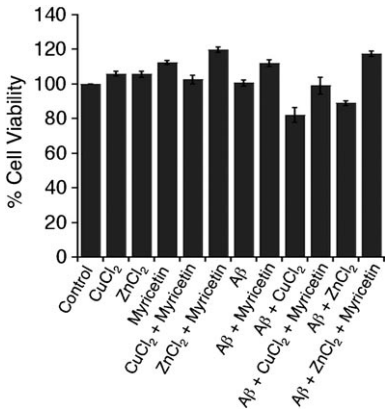

Figure 4. Regulation of metal-associated Aβ neurotoxicity in human neuroblastoma SK-N-BE(2)-M17 cells by myricetin. Cell survival was measured 24 h after incubation of myricetin in the presence of metal chloride salts and Aβ by using the MTT assay. The cell viability (%) depicted in the figure was calculated relative to that of cells containing only DMSO (1%, *v/v*; control). Experimental conditions: [Aβ] = 10 μM, [$CuCl_2$ or $ZnCl_2$] = 10 μM, [myricetin] = 20 μM. Values represent the mean of four independent experiments (± standard error).

measured by using the MTT assay.[12,13] In addition, cells incubated with only Aβ (10 μM) did not display cytotoxicity, while the treatment of cells with Aβ (10 μM) and $CuCl_2$ or $ZnCl_2$ (10 μM) for 24 h reduced cell survival (82(±4.3)% and 89(±1.4)%, respectively; Figure 4). The cytotoxicity of Aβ incubated with metal chloride salts occurred even in the presence of bovine serum albumin (BSA), a known metal chelator and a major component of FBS; this demonstrates that Aβ can compete for metal binding under these conditions, which can result in the detected cytotoxicity.[29–31]

The observed neurotoxicity imparted by Aβ in the presence of $CuCl_2$ or $ZnCl_2$ was recovered upon incubation with myricetin (20 μM) for 24 h. Myricetin regulated toxicity to different degrees in the cells depending on the metal ion present, a similar trend to the metal dependence in the aggregation studies. For the cells containing Aβ, Cu^{II}, and myricetin, ca. 100% of the cells survived, while cell viability was increased to over 100% for the Zn^{II} samples. In these cell results, myricetin could control metal–Aβ neurotoxicity, although the variance in the cell survival rate, depending on the metal ion, suggests its involvement in neuroprotection via more than one route. While the mechanism of neurotoxicity recovery is not known in our system, one potential explanation for these data might be related to recent in vitro and ex vivo studies that have indicated enhanced antioxidant capacities for metal–flavonoid complexes over the free flavonoid ligand.[32–35] Potentially, uncovering details of the neuroprotective function of myricetin could be valuable in its further application.

Considering that metal ions are found in Aβ plaques in diseased AD brains, identification of small molecules as chemical tools to study the relationship between metal ions, Aβ, and AD are desired. Myricetin is a naturally occurring flavonoid that can chelate metal ions and show anti-amyloidogenic reactivity toward Aβ; however, the influence of myricetin on metal-involved Aβ aggregation and neurotoxicity has not been studied

previously. Our investigations here demonstrate that myricetin could modulate metal-induced Aβ aggregation more effectively than metal-free Aβ aggregation. Myricetin also exhibited differential control on regulating Aβ aggregation pathways involving Cu^{II} over Zn^{II}. Furthermore, in living cells, myricetin diminished the cytotoxicity of Aβ treated with metal ions affording greater cell survival rates. Overall, to the best of our knowledge, the observations and results in this work are the first to demonstrate the reactivity of myricetin toward metal-associated Aβ species. Moreover, our studies suggest the high potential of using the framework of the flavonoid family to develop a new class of bifunctional molecules as chemical reagents for targeting and modulating metal–Aβ species that have been suggested to be key players in AD neuropathogenesis.

Experimental Section

Figure S1 in the Supporting Information and details describing the chemical reagents and methods, UV/Vis studies, reactivity of myricetin with Aβ species by using native gel electrophoresis and TEM, and cytotoxicity studies are available in the Supporting Information.

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Keywords: Alzheimer's disease · amyloid beta-peptides · flavonoids · metal ions · bifunctionality

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