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Letter

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Orobol: An Isoflavone Exhibiting Regulatory Multifunctionality against Four Pathological Features of Alzheimer's Disease

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ABSTRACT: We report **orobol** as a multifunctional isoflavone with the ability to (i) modulate the aggregation pathways of both metal-free and metal-bound amyloid-β, (ii) interact with metal ions, (iii) scavenge free radicals, and (iv) inhibit the activity of acetylcholinesterase. Such framework with multifunctionality could be useful for developing chemical reagents to advance our understanding of multi-faceted pathologies of neurodegenerative disorders, including Alzheimer's disease.

KEYWORDS: *Multifunctionality, flavonoid, amyloid-β, metal-amyloid-β, free radicals, acetylcholinesterase*

Alzheimer's disease (AD) is a progressive neurodegenerative disease responsible for the majority of dementia cases.[1,2] The lack of an effective cure and aging world population depict an imminent epidemic and pessimistic outlook. Although research efforts have been dedicated towards understanding the pathology of AD, our comprehension of the disease is fragmental and the principal causative factors of the disease remain unidentified.[1] Attempts to pinpoint the primary culprit behind AD have led to the proposal of several hypotheses (e.g., amyloid cascade, metal ion, oxidative stress, and cholinergic).[3–6] Clinical failures of drugs formulated based on the individual pathogenic targets [i.e., amyloid-β (Aβ), metal ions, free radicals, and acetylcholinesterase (AChE)] implicated in the aforementioned hypotheses have prompted a shift in paradigm to elucidate their malignant connections.[1] For instance, the intimate association between Aβ and metal ions [e.g., Cu(II) and Zn(II)] have led to the introduction of metal-bound Aβ (metal–Aβ) as a hybrid pathogenic component in AD.[7–9] Identification and evaluation of such pathological relations in AD present experimental challenges arising from the complexity involving multiple intricate biological components under physiologically relevant conditions. Thus, chemical tools capable of interacting with multiple pathological elements of AD could significantly expedite our efforts to elucidate the relationships between them. As candidates of such

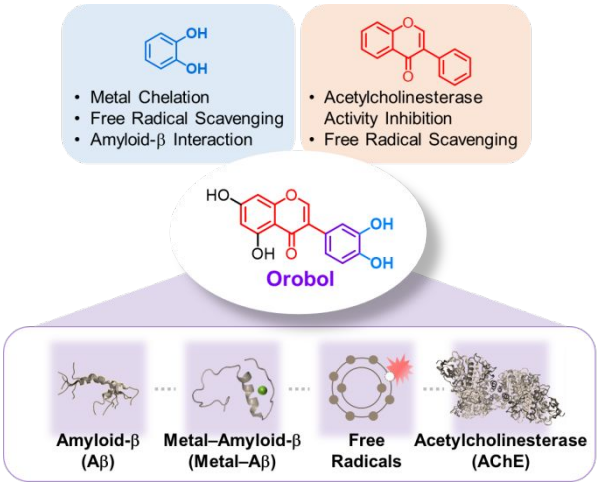
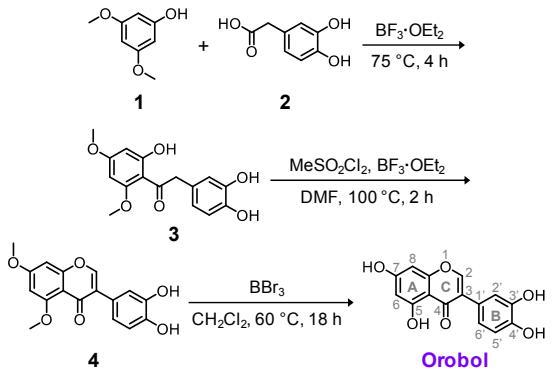


Figure 1. Rational identification of **orobol** as a multifunctional small molecule for regulating four pathological factors implicated in AD (i.e., metal-free Aβ, metal–Aβ, free radicals, and AChE).

chemicals, flavonoids, a naturally occurring family of polyphenols, have reportedly exhibited a multitude of chemical and biological activities (e.g., antioxidant,[10] antimicrobial,[11] antiviral,[12] and cytoprotective[12]).[13] Numerous compounds have been tested as chemical tools to understand AD pathology;[14–24] however, examples of

flavonoids capable of regulating four or more pathological factors found in AD are very limited.



Scheme 1. Synthetic routes to **orobol**.

Herein, we report, for the first time, a multifunctional isoflavone (**orobol**, Figure 1) capable of modulating four pathological targets (i.e., metal-free Aβ, metal–Aβ, free radicals, and AChE), to the best our knowledge. **Orobol** is characterized by an isoflavone framework with structural variations from the basic flavone backbone (Figure 1 and Scheme 1): (i) four hydroxyl substituents; (ii) catechol functionality on the B-ring; (iii) translocation of the B-ring. **Orobol** manifests two potential metal chelation sites (i.e., 3'-OH/4'-OH and 4-oxo/5-OH), suggesting its ability to interact with metal ions by forming a 5- or 6-membered ring.[17,18] The B-ring catechol moiety is a source of antioxidant activity with radical stabilization capabilities via intramolecular hydrogen bonding.[25,26] Oxidation of "catechol-type flavonoids" can produce an ortho-quinone that covalently binds to primary amine-containing residues of Aβ (e.g., K16 and K28).[27] Previous research regarding synthetic aminoisoflavones demonstrated the pertinence of both the catechol functionality and isoflavone framework towards their ability to modify the aggregation of Aβ.[17] Although **orobol**'s effect against AChE has not been directly reported, structure-activity relationship studies of flavonoids' ability to suppress the activity of AChE indicate the inhibitory potency of isoflavones and the significance of the catechol functional group.[28] Despite the collective implications for the versatility of **orobol** from previous studies, the multifunctionality of the compound against four pathological factors linked to AD pathology (i.e., metal-free Aβ, metal–Aβ, free radicals, and AChE) has not been directly evaluated until now.

RESULTS AND DISCUSSION

Orobol was prepared following previously reported procedures with minor modifications as shown in Scheme 1.[29,30] An electrophilic aromatic substitution reaction (Friedel-crafts acylation) of **1** with **2** produced **3** (17% yield) using boron trifluoride ethyl etherate ($BF_3{\cdot}OEt_2$) as both the solvent and catalyst.[29] The subsequent cyclization of **3** in the presence of methanesulfonyl chloride ($MeSO_2Cl_2$) generated **4** in 19% yield. Lastly, the demethylation of **4** with boron tribromide (BBr_3) afforded **orobol** (10% yield; Figures S1 and S2).[30] **Orobol** was predicted to exhibit borderline blood-brain barrier (BBB) permeability with a $-\log P_e$ value of 5.35 (± 0.06) by the parallel artificial membrane permeability assay adapted for the BBB (Table S1).

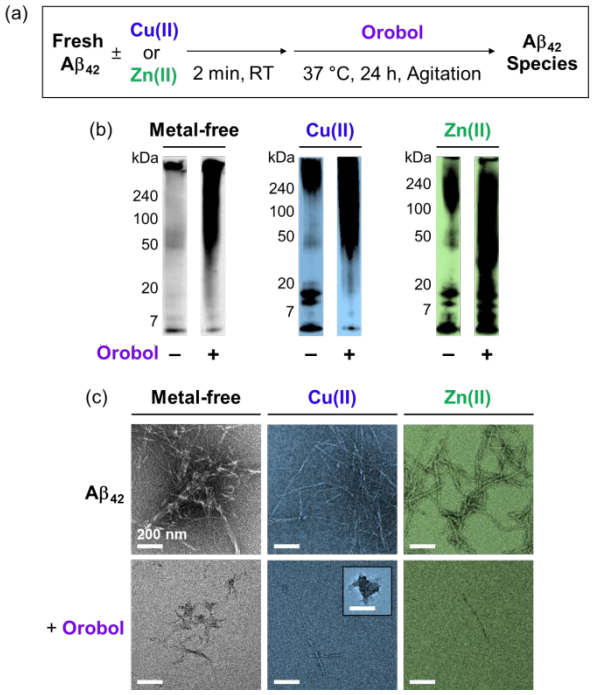


Figure 2. Influence of **orobol** on the formation of metal-free and metal-induced $Aβ_{42}$ aggregates. (a) Scheme of the inhibition experiment. (b) Analysis of the MW distribution of the resultant $Aβ_{42}$ species by gel/Western blot using an anti-Aβ antibody (6E10). The original gel images are presented in Figure S4. (c) TEM images of the samples from (b). Conditions: [$Aβ_{42}$] = 25 μM; [$CuCl_2$ or $ZnCl_2$] = 25 μM; [**orobol**] = 50 μM; pH 7.4 [for metal-free and Zn(II)-containing samples] or pH 6.6 [for Cu(II)-added samples]; 37 °C; 24 h incubation; constant agitation.

Orobol's effects on the aggregation pathways of metal-free $Aβ_{42}$ and metal–$Aβ_{42}$ were first determined through gel electrophoresis with Western blotting (gel/Western blot) in both inhibition (inhibition of $Aβ_{42}$ aggregate formation; Figure 2a) and disaggregation (disassembly and/or aggregation of preformed $Aβ_{42}$ aggregates; Figure S3a) experiments. In the inhibition experiments, **orobol** noticeably altered the molecular weight (MW) distribution of metal-free $Aβ_{42}$ species by increasing the intensity of the smearing bands greater than ca. 20 kDa compared to **orobol**-free $Aβ_{42}$ (Figure 2b; left). **Orobol**-treated Cu(II)–$Aβ_{42}$ exhibited darker bands at ca. 20-240 kDa and less intense bands at ca. 7-20 kDa, relative to Cu(II)–$Aβ_{42}$ without treatment of the compound. In the presence of Zn(II), **orobol** enhanced the band intensity at ca. 7-100 kDa. In addition to the inhibition studies, the disaggregation experiments illustrated the reactivity of **orobol** towards preformed aggregates of metal-free $Aβ_{42}$

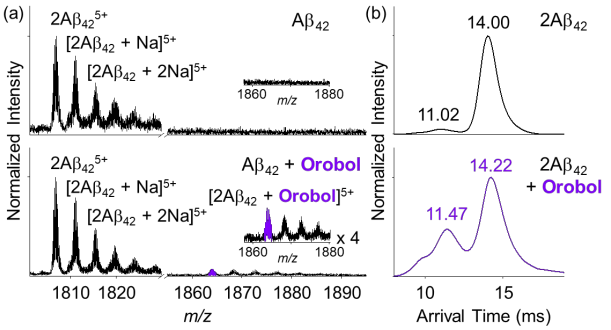


Figure 3. Interaction of **orobol** with metal-free $A\beta_{42}$. (a) Interaction of **orobol** with soluble metal-free $A\beta_{42}$, monitored by ESI-MS. Inset: Zoom-in spectrum from 1860 to 1880 *m/z* (indication of $[2A\beta_{42}$ + **orobol**$]^{5+}$ at 1863 *m/z*). (b) Analysis of the IM-MS spectra of $[2A\beta_{42}]^{5+}$ (black) and $[2A\beta_{42}$ + **orobol**$]^{5+}$ (purple). Conditions: $[A\beta_{42}]$ = 20 μM; [**orobol**] = 100 μM; 100 mM ammonium acetate, pH 7.4; 3 h incubation; no agitation.

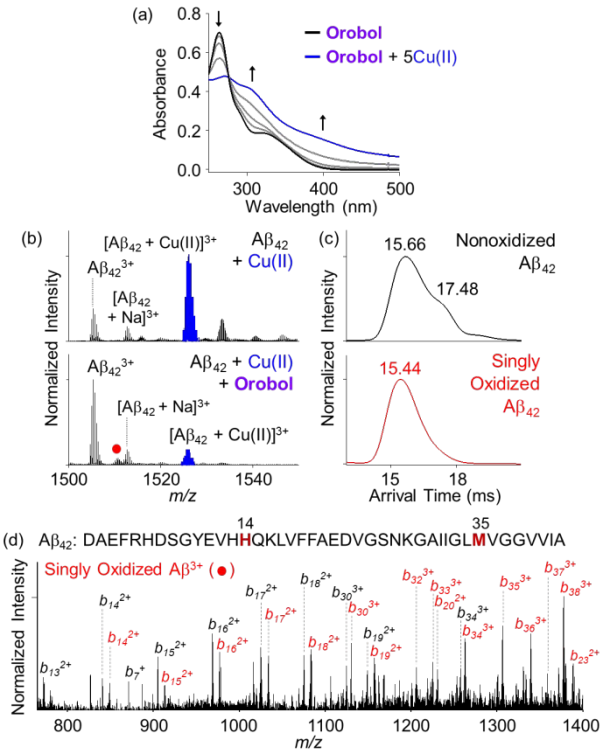


Figure 4. Interactions of **orobol** with Cu(II) and Cu(II)–$A\beta_{42}$. (a) Cu(II) binding of **orobol** in a buffered solution. UV–Vis spectra of **orobol** (black) with up to 5 equiv of Cu(II) (blue). Conditions: [**orobol**] = 25 μM; $[CuCl_2]$ = 0, 12.5, 25, 50, and 125 μM; pH 7.4; room temperature. Interaction of orobol with soluble Cu(II)–$A\beta_{42}$, monitored by (b) ESI-MS and (c) IM-MS. (d) Tandem mass spectrometric analysis in conjunction with CID of the singly oxidized $A\beta_{42}$ (●, at 1511 *m/z*). Conditions: $[A\beta_{42}]$ = 20 μM; $[CuCl_2]$ = 20 μM; [**orobol**] = 100 μM; 100 mM ammonium acetate, pH 7.4; 3 h incubation; no agitation.

and metal–$A\beta_{42}$ (Figure S3). Under metal-free conditions, when preformed $A\beta_{42}$ aggregates were incubated with **orobol**, a different MW distribution from **orobol**-free $A\beta_{42}$ aggregates with darker bands at ca. 50-240 kDa was observed (Figure S3b). **Orobol** noticeably reduced the band intensity of preformed Cu(II)–$A\beta_{42}$ aggregates smaller than at ca. 100 kDa. As for Zn(II)–$A\beta_{42}$, the MW distribution of preformed species was altered by **orobol** with an increase in the intensity of the smearing band at ca. 20-50 kDa. It should be noted that the Aβ aggregate species detected through gel/Western blot are small enough to enter the gel matrix. In order to visualize the larger Aβ assemblies, transmission electron microscopy (TEM) was used to examine the morphology of the resultant metal-free $A\beta_{42}$ and metal–$A\beta_{42}$ aggregates from the gel/Western blot experiments (Figures 2c and S3c). According to the TEM images, **orobol** discernibly modified the morphology of metal-free $A\beta_{42}$ and metal–$A\beta_{42}$ assemblies from both the inhibition and disaggregation experiments, inducing the formation of smaller chopped fibrils or amorphous aggregates. The modulative reactivity of **orobol** was also observed against $A\beta_{42}$ aggregation in the presence of Cu(I) and Fe(III) (Figure S5). Overall, the gel/Western blot and TEM results support **orobol**'s modulative reactivity towards the aggregation of both metal-free and metal-bound $A\beta_{42}$ species. Note that despite such reactivity, **orobol** could not noticeably reduce the toxicity of metal-free and metal-treated $A\beta_{42}$ in living cells under our experimental conditions (Figure S6). The cytotoxicity of **orobol** was minimal at concentrations up to 100 μM.

To elucidate the mechanistic details regarding **orobol**'s ability to modulate the aggregation pathways of metal-free $A\beta_{42}$ and metal–$A\beta_{42}$, spectrometric and spectroscopic studies [i.e., electrospray ionization-mass spectrometry (ESI-MS), tandem MS (ESI-MS^2), and UV–Visible spectroscopy (UV–Vis)] were performed to determine the interactions of **orobol** with metal-free $A\beta_{42}$, Cu(II), and Cu(II)–$A\beta_{42}$ (Figures 3 and 4). Under metal-free conditions, a $2A\beta_{42}$–**orobol** complex was detected (Figure 3a; purple). The complexation of **orobol** with $A\beta_{42}$ dimer was observed to vary the size distribution of the peptide dimer indicating a structural compaction, according to the ion mobility-mass spectrometry (IM-MS) data (Figure 3b; purple). Such changes could be responsible for the altered aggregation pathways of $A\beta_{42}$.[17,31]

Interaction between Cu(II) and **orobol** was confirmed via UV–Vis denoted by notable optical changes with the addition of Cu(II) to an aqueous solution of the isoflavone (Figure 4a). Under Cu(II)-present conditions, the mass spectrometric analysis of **orobol**-treated $A\beta_{42}$ led to the detection of singly oxidized $A\beta_{42}$ (Figure 4b; red circle). The oxidation-induced conformational change of $A\beta_{42}$ monomer was monitored by IM-MS (Figure 4c), in which the singly oxidized $A\beta_{42}$ exhibited a more compact structure. Using ESI-MS^2, the oxidation sites of $A\beta_{42}$ were determined by analyzing the peptide fractions of the singly oxidized $A\beta_{42}$, reported as *b* fragments (Figure 4d). Upon collision-induced dissociation (CID) of $[A\beta_{42}$ + O$]^{3+}$ at 1511 m/z, Aβ fragments were found in their oxidized form from b_{14}, revealing H14 as a possible oxidation site. Based on the indication of a mixture containing both the oxidized and

(a)

	Trolox	Orobol
TEAC Value	1.00 ± 0.04	3.39 ± 0.14

	Tacrine	Orobol
AChE IC_{50} (nM)	38.7 ± 5.0	123 ± 32

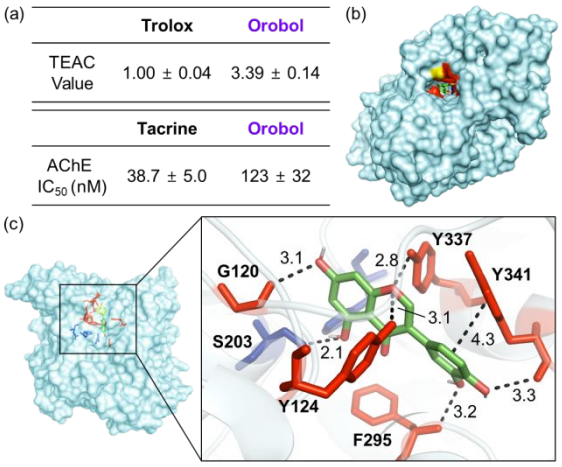


Figure 5. Free radical scavenging and AChE inhibitory activities of **orobol**. (a) TEAC values and IC_{50} values against AChE activity of **orobol** and the corresponding standards (Trolox: antioxidant; Tacrine: AChE inhibitor).[32] (b,c) Docking studies of **orobol** and AChE (PDB 1C2B[33]). (b) Top view perspective looking into the active site gorge of AChE. (c) Side view of **orobol** bound to AChE and zoom-in image of the interaction between **orobol** and the amino acid residues of the active site gorge. The dashed lines and the corresponding numbers indicate the possible hydrogen bonding between **orobol** and AChE and their predicted distances (Å).

non-oxidized fragments from b_{14} to b_{34} and the sole detection of the oxidized Aβ fragments starting from b_{35}, M35 was designated as another plausible oxidation site of Aβ. A noteworthy observation was that **orobol** noticeably diminished the peak intensity of Cu(II)–$A\beta_{42}$ while increasing that of metal-free $A\beta_{42}$ (Figure 4b), indicating that the isoflavone may disrupt the interaction between $A\beta_{42}$ and Cu(II), potentially through Cu(II) chelation.[18] Together, our spectrometric and spectroscopic studies of **orobol** demonstrate its ability to (i) interact with metal-free $A\beta_{42}$ and Cu(II), (ii) disrupt the interaction between Cu(II) and $A\beta_{42}$, and (iii) oxidize $A\beta_{42}$ in the presence of Cu(II) at H14 or M35. Such molecular interactions and alterations of Aβ have previously been reported as mechanistic strategies to modify the aggregation pathways of $A\beta_{42}$ in both the absence and presence of metal ions.[34–37] Note that Zn(II)–$A\beta_{42}$ could not be detected under our experimental conditions for ESI-MS studies.

The free radical scavenging activity of **orobol** was determined relative to Trolox, a vitamin E analog with notable antioxidant activity, using the Trolox equivalent antioxidant capacity (TEAC) assay (Figure 5a).[38] **Orobol** was observed to significantly scavenge free radicals with a TEAC value of 3.39 (± 0.14). Moreover, **orobol**'s inhibitory activity against AChE was measured by a fluorometric AChE assay,[39] indicating a nanomolar IC_{50} value [123 (± 32) nM; Figure 5a]. In silico studies (Figure 5b and c) presented that **orobol** could interact with multiple amino acid residues lining the active site gorge (e.g., G120, Y124, F295, Y337, and Y341). **Orobol** was shown to interact with S203 of the catalytic triad, responsible for initiating the enzymatic hydrolysis of acetylcholine (ACh),[40] via hydrogen bonding, suggesting a potential mechanism for **orobol**'s inhibitory activity against AChE. The interactions between **orobol** and AChE were further analyzed by ^{1}H NMR (800 MHz; Figure S7). The peak intensities of **orobol** were noticeably decreased upon titration with AChE. These NMR results suggest a significant intermolecular interactions between **orobol** and AChE.

CONCLUSION

Orobol, a naturally occurring isoflavone, is demonstrated as a multifunctional small molecule able to modulate four distinct pathological features found in the brains affected by AD (i.e., metal-free and metal-induced Aβ aggregation, oxidative stress, and AChE-catalyzed ACh hydrolysis). Aside from the properties tested herein, additional neuroprotective properties of **orobol** have been previously reported: (i) protection against 6-hydroxydopamine-induced neurotoxicity by restoring proteasomal function;[41] (ii) anti-inflammatory effects;[42] (iii) inhibition of tyrosine-specific protein kinase;[43] phosphatidylinositol turnover;[44] and 15-lipoxygenase;[45] (iv) hypotensive effects.[46] Based on its functional versatility, the isoflavone framework of **orobol** conferring multifunctionality could be useful towards developing chemical reagents for advancing our understanding of the multi-faceted pathology of neurodegenerative disorders, including AD.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Experimental details, Table S1, and Figures S1-S7 (PDF).

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Notes

The authors declare no competing financial interest.

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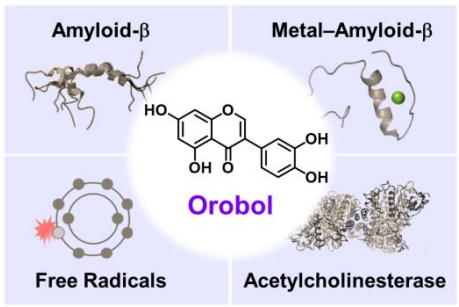
TOC


Amyloid-β
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Acetylcholinesterase