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Curcumin/Melatonin Hybrid 5-(4-Hydroxy-phenyl)-3-oxo-pentanoic Acid [2-(5-Methoxy-1*H*-indol-3-yl)-ethyl]-amide Ameliorates AD-Like Pathology in the APP/PS1 Mouse Model

Gorka Gerenu,[†,#] Kai Liu,[§,#] Jeremy E. Chojnacki,[§] John M. Saathoff,[§] Pablo Martínez-Martín,[∥] George Perry,[⊥] Xiongwei Zhu,[†,‡] Hyoung-gon Lee,*[,†,‡] and Shijun Zhang*[,§]

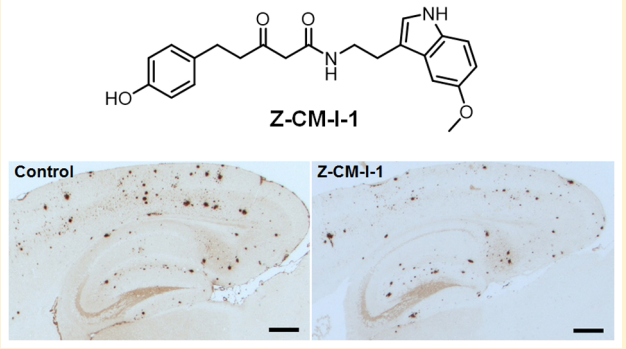
Departments of [†]Pathology and [‡]Neurology, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106, United States

[§]Department of Medicinal Chemistry, Virginia Commonwealth University, Richmond, Virginia 23298, United States

[∥]Alzheimer Disease Research Unit, Alzheimer Center Reina Sofía Foundation, CIEN Foundation, Carlos III Institute of Health, 28071 Madrid, Spain

[⊥]College of Sciences, University of Texas at San Antonio, San Antonio, Texas 78249, United States

ABSTRACT: In our efforts to develop hybrid compounds of curcumin and melatonin as potential disease-modifying agents for Alzheimer's disease (AD), a potent lead hybrid compound, **Z-CM-I-1**, has been recently identified and biologically characterized *in vitro*. In this work, we report the *in vivo* effects of **Z-CM-I-1** on AD pathologies in an APP/PS1 transgenic AD model. Our studies demonstrated that **Z-CM-I-1** significantly decreased the accumulation of Aβ in the hippocampus and cortex regions of the brain and reduced inflammatory responses and oxidative stress after treatment for 12 weeks at 50 mg/kg per dose via oral administration. Furthermore, **Z-CM-I-1** significantly improved synaptic dysfunction evidenced by the increased expression of synaptic marker proteins, PSD95 and synaptophysin, indicating its protective effects on synaptic degeneration. Lastly, we demonstrated that **Z-CM-I-1** significantly increased the expression level of complexes I, II, and IV of the mitochondria electron transport chain in the brain tissue of APP/PS1 mice. Collectively, these results clearly suggest that **Z-CM-I-1** is orally available and exhibits multifunctional properties *in vivo* on AD pathologies, thus strongly encouraging further development of this lead compound as a potential disease-modifying agent for AD patients.



KEYWORDS: *Hybrid compounds, neuroprotectants, curcumin, melatonin, Alzheimer's disease*

Alzheimer's disease (AD) is a devastating neurodegenerative disorder affecting around 5.2 million Americans.[1] Multiple pathological factors have been extensively studied to understand the underlying mechanisms of AD.[2−5] However, the exact etiology of AD still remains unknown. Addressing the issue of the paucity of effective therapeutics in the pipeline and taking the multifaceted nature of AD into account, drug development efforts have been devoted to multifunctional compounds that can target more than one potential risk factor simultaneously, thus increasing the success of disease-modifying agents for AD.[6,7]

Recently, we have begun development of hybrid compounds from the structures of curcumin and melatonin, two natural products that have shown therapeutic potential in various AD models, with the intention of providing novel chemical scaffolds with potentially new or different mechanisms of action. Our results demonstrated that the hybrid strategy is a viable approach in providing chemotypes with novel pharmacology, and one lead compound, **Z-CM-I-1**, has been identified for further optimization and validation (Figure 1).[8] Preliminary mechanistic studies revealed that this lead compound might interfere with the interactions between amyloid-β (Aβ) and mitochondria in order to exert its neuroprotective activities. More importantly, we have demonstrated that **Z-CM-I-1** can efficiently penetrate the blood−brain barrier after oral administration.[8] Herein, we report that the treatment of **Z-CM-I-1** in the APP/PS1 transgenic mouse model[9] significantly reduces multiple pathologies found in AD.

RESULTS AND DISCUSSION

Z-CM-I-1 Decreases Aβ Burden in APP/PS1 Mice after Long-Term Treatment. Aβ plaques have been recognized as one of the hallmarks of AD patients.[2,10] It has also been

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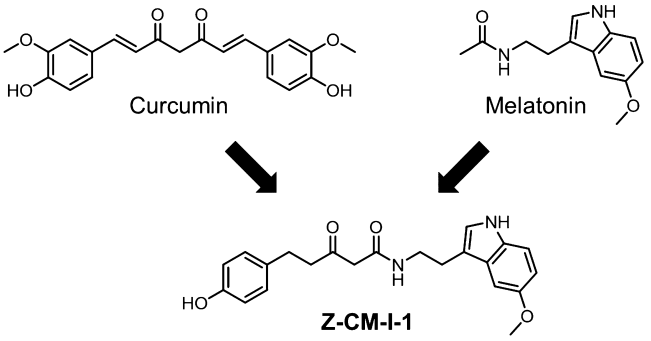

Figure 1. Structure of hybrid compound **Z-CM-I-1** and the natural products from which it is derived, curcumin and melatonin.

demonstrated that Aβ plaque load is increased in the aging APP/PS1 mouse model.[9] Therefore, we first examined the effects of **Z-CM-I-1** (50 mg/kg) on Aβ accumulation in the brain after treatment. After quantification and data analysis (see Methods, Aβ quantification), treatment with **Z-CM-I-1** was shown to significantly decrease the level of Aβ plaques in the cerebral cortex and hippocampal areas ($^*p < 0.05$) (Figure 2) compared to that in the nontreated group. From our previous *in vitro* studies, we demonstrated that **Z-CM-I-1** shows minimal inhibition of Aβ aggregation, whereas it moderately inhibits the production of small Aβ oligomers (AβOs) in MC65 cells.[8] This suggests that the reduction of Aβ plaque load in APP/PS1 mice could be a main downstream outcome of the interference of **Z-CM-I-1** on other factors.

Z-CM-I-1 Reduces Activation of Microglia in APP/PS1 Mice. A growing body of evidence has implicated neuroinflammation as an essential player in the etiology of AD.[3,11] This notion is supported by a number of epidemiological studies showing evidence that levels of inflammatory proteins, including C-reactive protein and inflammatory cytokines, are elevated long before the clinical symptoms of AD.[12,13] Furthermore, clinical trials have provided evidence that long-term use of nonsteroidal anti-inflammatory drugs can prevent or delay the onset of AD, especially when applied during early and asymptomatic phases of the disease.[14−16] Pathologically, activated microglia, along with elevated proinflammatory cytokines, have been observed in AD animal models and patients.[17−19] Notably, curcumin and melatonin have been reported to exhibit anti-inflammatory effects in a variety of disease models.[20−23] To explore whether **Z-CM-I-1** preserves the anti-inflammatory properties of curcumin and melatonin, we next studied the status of neuroinflammation in APP/PS1 mice after long-term treatment with **Z-CM-I-1**. Since microglia are a main neuroinflammatory cell type, which has been widely used as a marker for neuroinflammation, we therefore checked the activation of microglial cells by immunocytochemistry with an anti-Iba1 antibody, shown in Figure 3. While no change was detected in total microglial cell number, when microglial cells were further classified morphologically (Figure 3), treatment with **Z-CM-I-1** significantly decreased the population of type IV glial cells, which are the most activated glial cell type (Figure 3D). In the control group, type IV microglia cells were around 6% of total microglia, and the **Z-CM-I-1** treatment was able to reduce this significantly to 3% (Figure 3G). These results suggest that even though there were no differences between treated and nontreated groups in the total number of cells (Figure 3E), the microglia present at higher activation states were reduced in the **Z-CM-I-1**-treated group, thus suggesting an anti-inflammatory effect *in vivo*.

Z-CM-I-1 Reduces Oxidative Stress in APP/PS1 Mice after Long-Term Treatment. Mounting evidence from pharmacological and genetic studies has confirmed a correlation between oxidative stress and Aβ accumulation.[5,24−26] For example, Aβ can reduce redox-active metals to produce reactive oxygen species (ROS), which contribute to most types of oxidative damage noted in AD.[27−29] Furthermore, the interaction of microglia with Aβ results in increased ROS burden, further contributing to oxidative stress and neuroinflammation and eventually causing neurodegeneration.[30−33] To determine whether **Z-CM-I-1** can modulate oxidative stress

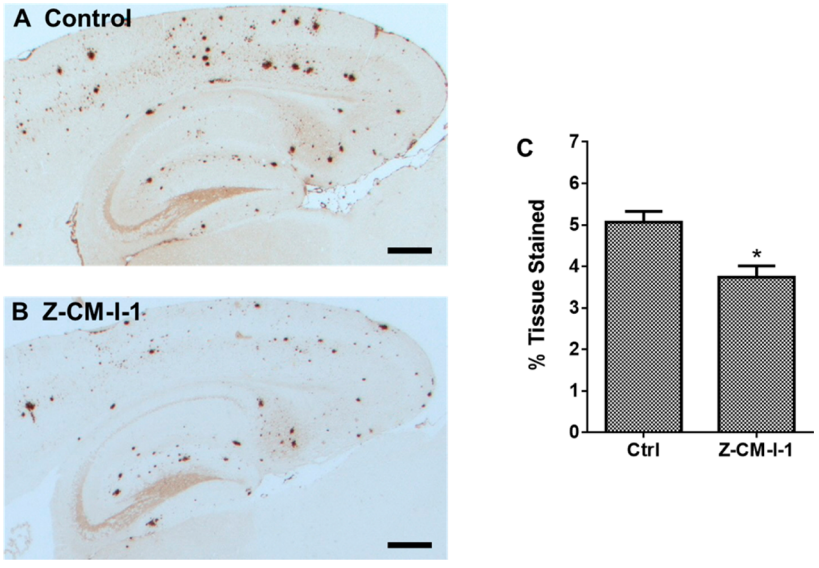

Figure 2. Aβ plaque load in treated versus control mice. **Z-CM-I-1** (50 mg/kg) was administered by oral gavage in 4 month old APP/PS1 transgenic animals for 12 weeks. Immunohistochemistry was performed using the anti-Aβ 82E1 antibody, and images of the cortex and hippocampus of control mice (A) and **Z-CM-I-1** treated mice (B) were analyzed. (C) The amount of Aβ plaque was quantified as percent area. **Z-CM-I-1** significantly reduced the total amount of Aβ plaques. Data are represented as mean ± SEM; $^*P < 0.05$. Scale bars represent 500 μm.

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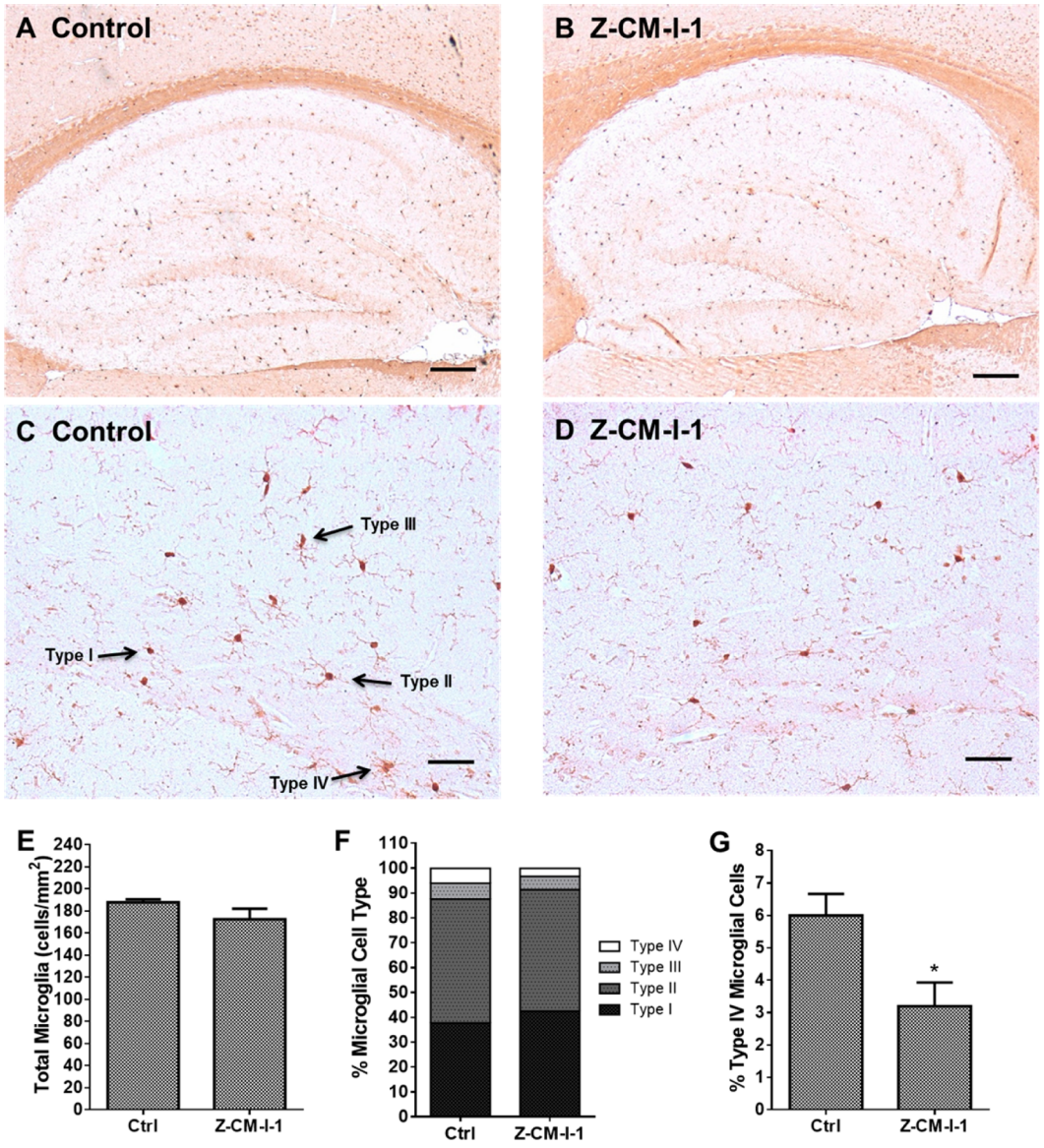

Figure 3. Effect of **Z-CM-I-1** on microglial cells. Immunohistochemistry was performed using the anti-Iba-1 antibody, and images of the hippocampus from (A, C) control mice (n = 4) and (B, D) **Z-CM-I-1** (n = 7) treated mice were analyzed. (E) The total number of microglial cells was quantified as cell number per square millimeter. There was no significant difference between control and treated mice. Data are represented as mean ± SEM. (C, D) Images show representative details of different microglial types (activation states). (F) Percentage of different microglial cell types were quantified by type classification (see Methods for a detailed explanation of this classification). (G) **Z-CM-I-1** significantly reduced the percentage of type IV microglial cells, the highest activation state. Data are represented as mean ± SEM; *P < 0.05. (A, B) Scale bars represent 200 μm. (C, D) Scale bars represent 50 μm.

in APP/PS1 mice, two oxidative stress markers were measured. As shown in Figure 4, long-term treatment with **Z-CM-I-1** resulted in a significant decrease of 8-hydroxyguanine (8OHG) (Figure 4A–C), a marker of oxidative damage on nucleic acids, in hippocampal neuronal nuclei. Notably, treatment of APP/PS1 mice with **Z-CM-I-1** also significantly reduced the level of 4-hydroxy-2-nonenal (HNE) (Figure 4D,E), a marker of lipid peroxidation. These results are consistent with our previously published *in vitro* results from MC65 cells demonstrating that lipid peroxidation is highly involved in the oxidative stress and cell death of MC65 cells and that **Z-CM-I-1** efficiently suppressed this oxidative stress.[8] Taken together, the results clearly show that **Z-CM-I-1** exhibits antioxidative effects in APP/PS1 mice after long-term treatment.

Treatment of APP/PS1 Mice with Z-CM-I-1 Improves the Level of Synaptic Markers. Synaptic dysfunction has been observed in AD patients and models and has been suggested to be an early event underlying AD progression.[34−37] Furthermore, synaptic loss correlates well with the severity of cognitive impairment.[38−40] Additionally, Aβ species, especially the small AβOs, have been shown to impair synaptic neurotransmission in various AD animal models.[41−44] To evaluate whether **Z-CM-I-1** has any effects on synapse pathology, we examined the expression levels of PSD95 and synaptophysin, postsynaptic and presynaptic markers, respectively. Notably, as shown in Figure 5, treatment of APP/PS1 mice with **Z-CM-I-1** resulted in a significant increase of PSD95 and synaptophysin compared to the nontreated group. From our previous *in vitro* studies, we have demonstrated that **Z-CM-**

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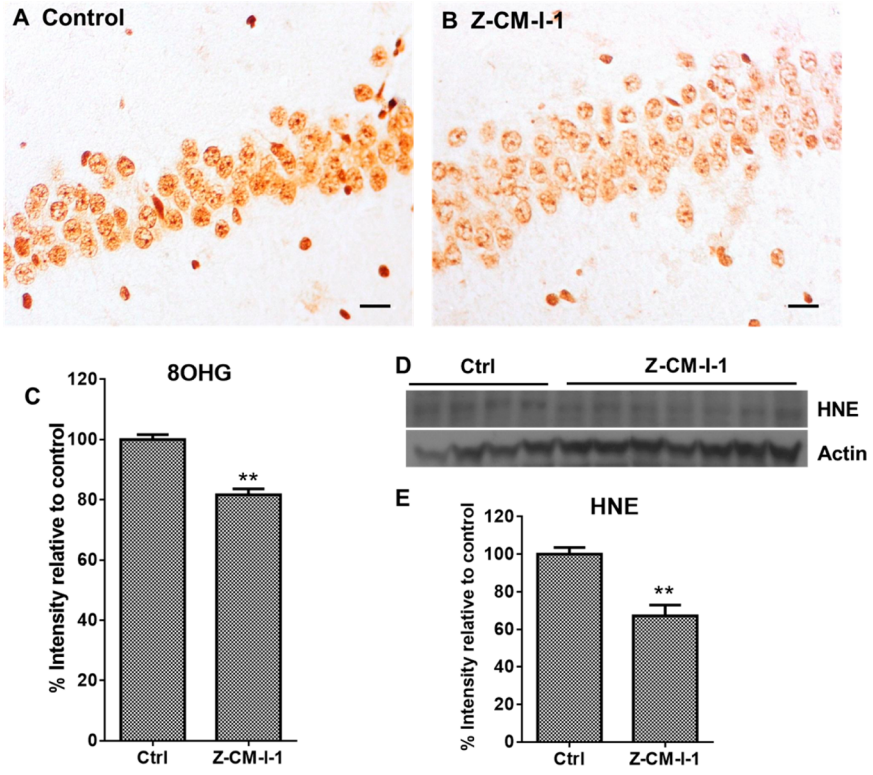

Figure 4. Effect of **Z-CM-I-1** on oxidative stress. (A, B) Immunohistochemistry was performed using the anti-8OHG antibody, an oxidative stress marker, and images of the hippocampus from (A) control mice (n = 4) and (B) **Z-CM-I-1** (n = 7) treated mice were analyzed. (C) Intensity of neuronal DNA damage in the CA1, CA2, and CA3 regions was measured and normalized to the control. **Z-CM-I-1** significantly reduced the extent of oxidative damage to neuronal nucleic acids. Data represent mean ± SEM; $^{**}P < 0.01$. Scale bars represent 20 μm. (D) Tissue homogenates from the cortex and hippocampus of control mice (n = 4) and **Z-CM-I-1** (n = 7) treated mice were prepared and subjected to western blotting using the anti-HNE antibody, an oxidative stress marker. (E) Band intensity was normalized to actin and quantified relative to control. **Z-CM-I-1** was able to significantly reduce the amount of HNE-protein adducts. Data represent mean ± SEM; $^{**}P < 0.01$.

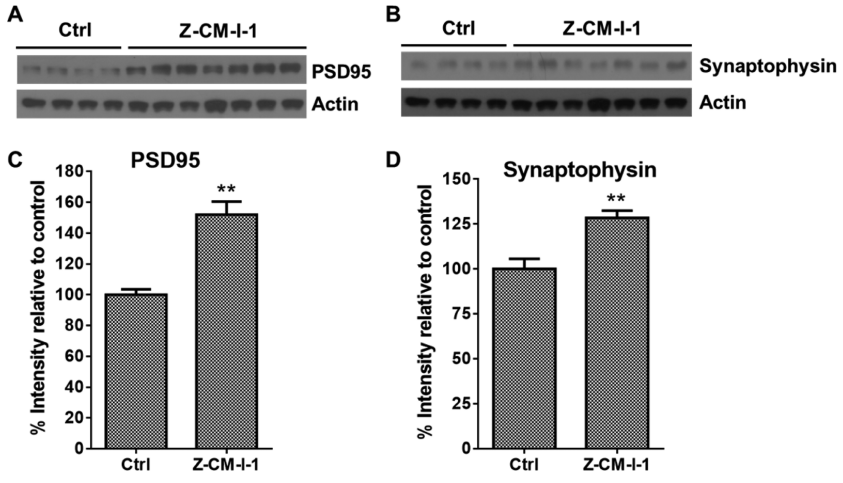

Figure 5. Effect of **Z-CM-I-1** on synaptic degeneration. (A, B) Tissue homogenates from the cortex and hippocampus of control mice (n = 4) and **Z-CM-I-1** (n = 7) treated mice were prepared and subjected to western blotting using anti-PSD95 and anti-synaptophysin antibodies. (C, D) Band intensity was normalized to actin and quantified relative to controls. **Z-CM-I-1** was able to significantly increase the amount of both PSD95 and synaptophysin in the cortex and hippocampus. Data represent mean ± SEM; $^{**}P < 0.01$.

I-1 moderately inhibits the formation of AβOs in MC65 cells and may interfere with the interactions of AβOs and their partner proteins.[8] Furthermore, we demonstrated that **Z-CM-I-1** treatment significantly reduced Aβ burden in the brain of APP/PS1 mice. Taken together, these effects on Aβ could potentially contribute to the observed synaptoprotective effects. Further studies are warranted to understand the mechanisms of **Z-CM-I-1** on synaptic function. Collectively, the results of **Z-CM-I-1** in APP/PS1 mice strongly suggest that it can improve synaptic degenerative phenotypes, suggesting its potential protective effect on cognitive deficit in AD.

Treatment of APP/PS1 Mice with Z-CM-I-1 Increases the Expression of Mitochondrial Electron Transport Chain Proteins. Mitochondria play critical roles in cellular

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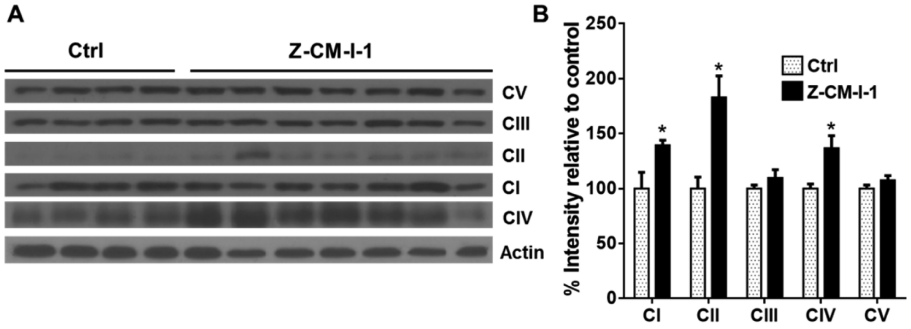

Figure 6. Effect of **Z-CM-I-1** on mitochondrial complex protein expression. (A, B) Tissue homogenates from the cortex and hippocampus of control mice (n = 4) and **Z-CM-I-1** (n = 7) treated mice were prepared and subjected to western blotting using the OXPHOS antibody cocktail, which detects complexes I, II, III, and V, and anticomplex IV antibody. (C, D) Band intensity was normalized to actin and quantified relative to controls. **Z-CM-I-1** significantly increased the expression of complexes I, II, and IV, but it did not affect complexes III and V. Data represent mean ± SEM; *P < 0.05.

functions, and various types of defects in mitochondria have been observed in AD patients and AD animal models.[4,30,45] In fact, APP/PS1 mice have been shown to exhibit deregulation in OXPHOS signaling, indicative of functional impairment of mitochondria.[46,47] Our previous results suggest that **Z-CM-I-1** may exert its neuroprotective effect through mitochondria.[8] Therefore, after long-term treatment, we further examined its effects on mitochondrial electron transport chain proteins by western blot analysis. As shown in Figure 6, treatment of APP/PS1 mice with **Z-CM-I-1** significantly increased the expression levels of complexes I, II, and IV. These results are consistent with our *in vitro* studies in MC65 cells suggesting that mitochondria might be the main organelle that **Z-CM-I-1** is targeting.[8] However, this does not rule out the possibility that the change in mitochondrial electron transport chain proteins could be a downstream outcome of the reduced Aβ accumulation in APP/PS1 mice. This could be due to either the direct interaction of **Z-CM-I-1** with the respiration chain proteins or the interference of **Z-CM-I-1** between Aβ species and the mitochondria. Further detailed mechanistic study is warranted to better understand the mechanisms of action of **Z-CM-I-1** in mitochondria.

In summary, we characterized the *in vivo* effects of **Z-CM-I-1** (50 mg/kg) on AD-related pathologies in APP/PS1 mice after long-term treatment via oral administration. The results revealed that **Z-CM-I-1** effectively reduced the Aβ burden, decreased the population of highly activated microglial cells, and ameliorated oxidative stress. Notably, **Z-CM-I-1** significantly improved synaptic degenerative changes, thus suggesting its therapeutic potential on memory and cognitive deficits in AD. All of the observed effects on AD pathologies might be due to its interactions at the mitochondria, as evidenced by its effects on complexes I, II, and IV of the electron transport chain. Collectively, the results from **Z-CM-I-1** treatment of APP/PS1 mice demonstrated that it exhibited multiple effects on Aβ and inflammatory responses as well as on oxidative stress, consistent with our original design rationale to provide novel chemical scaffolds by hybridization. These results also strongly encourage further characterization of **Z-CM-I-1** for its effects on cognition and behavioral function, as well as further optimization to develop more potent analogues.

METHODS

Animals and Z-CM-I-1 Treatment. Four month old double-transgenic female APP/PS1 mice expressing chimeric mouse/human amyloid precursor protein (Mo/HuAPP695swe) and the PS1-dE9 mutant human presenilin 1 were obtained from the Jackson Laboratory (B6C3-Tg(APPswe,PSEN 1dE9)85Dbo/J) and were administered either vehicle (n = 4) or **Z-CM-I-1** (50 mg/kg) (n = 7)[8] by oral gavage. Animals were treated five times per week for 12 consecutive weeks. All mice were maintained under an approved protocol in accordance with guidelines established by the Case Western Reserve University IACUC.

Tissue Collection. At the end of the treatment, all mice were sacrificed, and brain tissues were collected. Half sagittal brain hemispheres of each animal were immediately frozen and stored at −80 °C for biochemical analyses, and the other halves were fixed in 4% paraformaldehyde for immunohistochemical studies. Following fixation, tissue was dehydrated through ascending concentrations of ethanol and embedded in paraffin, and 6 μm sections were placed onto coated slides. For each animal, sections spanning the entire hippocampus were numbered and used for quantitative analysis.

Western Blot Assay. Brain tissue (cerebral cortex and hippocampus) was homogenized in cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) with added protease and phosphatase inhibitors (Roche). Homogenates were centrifuged at 14 000 g for 20 min at 4 °C. The supernatants were stored at −80 °C, and the proteins (20 μg per lane) were resolved by 10% SDS-PAGE. Proteins were transferred to Immobilon PVDF membranes (Millipore Corporation, Billerica, MA, USA) and blocked with 5% nonfat milk. The following primary antibodies were applied overnight at 4 °C: anti-synaptophysin (1:5000) (Abcam, Cambridge, UK), anti-HNE (1:1000) (Alpha Diagnostics), anti-PSD95 (1:2000) (Abcam, Cambridge, UK), mitochondrial complex OXPHOS (1:5000) (Abcam, Cambridge, UK), and anti-Complex IV (1:2000) (Abcam, Cambridge, UK). Then, the blots were rinsed in Tris-buffered saline (TBS) with 0.1% Tween 20 (TBS-T), and HRP-labeled rabbit or mouse secondary antibodies were applied (Cell Signaling Technology, Danvers MA, USA). Finally, membranes were rinsed in TBS-T, briefly incubated with a luminol reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and developed on X-ray film. For quantitative analysis, blots were scanned and analyzed with Quantity One image analysis software (BioRad). Actin levels were used as a loading control.

Immunohistochemistry. Tissue sections were deparaffinized in xylene and hydrated through descending ethanol concentrations, and endogenous peroxidase activity was inactivated by 30 min incubation in 3% hydrogen peroxide in methanol. For some experiments, antigen retrieval through pressure cooking was performed using the manufacturer's recommendations (Biocare Medical, Concord, MA, USA). Subsequently, in all cases, nonspecific binding sites were blocked with 30 min incubation in 10% normal goat serum (NGS) in TBS, and then sections were incubated overnight at 4 °C with the following primary antibodies: anti-Aβ antibody 82E1 (1:500, Immunobiological Laboratories, Minneapolis, MN, USA), anti-Iba-1 (1:250, Wako, Osaka, Japan), and anti-8OHG (1:1000, Thermo Scientific, Waltham, MA, USA). Species-specific secondary antibodies and PAP complexes were then added in sequence. Slides were

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developed with 3′-3′-diaminobenzidine (DAB, Dako), dehydrated through ascending ethanol to xylene, and then coverslipped. Images were obtained with a Zeiss Axiophot.

Microglial Cell Activation State Classification. Microglial cell morphological classification was manually measured by size, shape, and branch number[48,49] and defined as follows: Type I: Nonactivated state cells; without arms and circular shape. Nonramified microglia. Type II: Low activation state cells; 2−3 arms per cell, a circular-star shape; ramified microglia. Type III: Moderate activation state cells; 4−5 arms or more per cell and star-shaped; hypertrophied microglia. Type IV: Heavy activation state cells; bigger cell body with 5 or more arms and strongly star-shaped. Two tissue sections per animal were used to measure total glial cells in the hippocampus regions, and quantification was performed by Axiovision image analysis software (Zeiss).

Aβ Plaque Quantification and Data Analysis. For quantification of the amyloid plaque load in cerebral cortex and hippocampal areas, five tissue sections from each animal approximately 140 μm apart were immunostained with the 82E1 antibody. Each section contained a different population of plaques since the largest plaque measured was near 100 μm in diameter. Staining and quantification were performed in a blind fashion. For image analysis, using a 2.5× objective, the entire hippocampus and cortex were imaged. The regions of interest were cropped out, carefully omitting large amyloid containing vessels near the ventricle and along the meningeal layer. Finally, the percent area immunostained was determined from the cropped images using Axiovision software, setting a minimum threshold size to mark only the smallest visible plaque deposition. All images were analyzed with the software under the same conditions and at the same time. The mean values for all five sections were determined for each animal. Finally, the mean ± SEM was calculated for both groups.

Statistical Analysis. The Student's *t* test was used for all statistical analysis. Data are presented as mean ± SEM. The level of significance for all analysis testing was set at $^{*}P < 0.05$.

AUTHOR INFORMATION

Corresponding Authors

*(H.-g.L.) E-mail: hyoung-gon.lee@case.edu. Phone: 216-368-6887.

*(S.Z.) E-mail: szhang2@vcu.edu. Phone: 804-628-8266. Fax: 804-828-7625.

Author Contributions

#G.G. and K.L. contributed equally to this work. Chemical synthesis was completed by K.L. and J.M.S.; animal studies were completed by G.G. and H.-g.L.; data analysis was completed by G.G., K.L., J.E.C., P.M.-M., G.P., X.Z., H.-g.L., and S.Z.; experiment design was completed by G.G., H.-g.L., and S.Z.; writing and editing were completed by G.G., K.L., J.E.C., H.-g.L., and S.Z.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

Aβ, amyloid-β; AβOs, amyloid-β oligomers; AD, Alzheimer's disease; HNE, 4-hydroxy-2-nonenal; NGS, normal goat serum; 8OHG, 8-hydroxyguanine; ROS, reactive oxygen species; TBS, Tris-buffered saline

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