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Discovery of 5-(4-Hydroxyphenyl)-3-oxo-pentanoic Acid [2-(5-Methoxy-1H-indol-3-yl)-ethyl]-amide as a Neuroprotectant for Alzheimer's Disease by Hybridization of Curcumin and Melatonin

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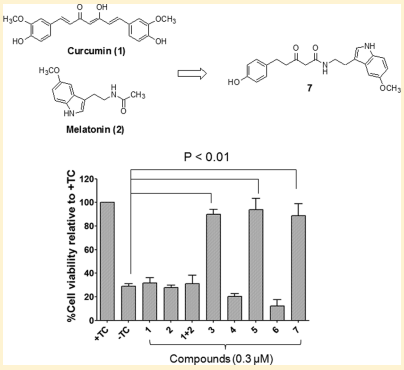
Supporting Information

ABSTRACT: In our effort to develop effective neuroprotectants as potential treatments for Alzheimer's disease (AD), hybrid compounds of curcumin and melatonin, two natural products that have been extensively studied in various AD models, were designed, synthesized, and biologically characterized. A lead hybrid compound (**7**) was discovered to show significant neuroprotection with nanomolar potency (EC_{50} = 27.60 ± 9.4 nM) in MC65 cells, a cellular AD model. Multiple in vitro assay results established that **7** exhibited moderate inhibitory effects on the production of amyloid-β oligomers (AβOs) in MC65 cells, but not on the aggregation of Aβ species. It also exhibited significant antioxidative properties. Further mechanistic studies demonstrated that **7**'s antioxidant effects correlate well with its neuroprotective potency for MC65 cells, and these effects might be due to its interference with the interactions of AβOs within the mitochondria of MC65 cells. Furthermore, **7** was confirmed to cross the blood-brain barrier (BBB) and deliver a sufficient amount to brain tissue after oral administration. Collectively, these results strongly support the hybridization approach as an efficient strategy to help identify novel scaffolds with a desired pharmacology, and strongly encourage further optimization of **7** to develop more potent neuroprotectants for AD.



KEYWORDS: *Neuroprotectants, hybridization, Alzheimer's disease, curcumin, melatonin*

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the most common cause of dementia. It is estimated that 5.2 million Americans of all ages and up to 30 million individuals worldwide are affected by AD.[1] Current AD treatments provide mainly symptomatic relief, and there are no agents available to delay or cure this disease. Although significant advances have been made in understanding the mechanisms leading to AD, the exact etiology of AD still remains elusive and multiple factors, including amyloid-β (Aβ) aggregates,[2] soluble Aβ oligomers (AβOs),[3−5] dyshomeostasis of biometals, oxidative stress, and neuroinflammation,[6,7] have been implicated in the development of AD. Recently, the multifunctional strategy of small molecule design has attracted extensive attention in overcoming the limitations of the traditional "one molecule, one target" approach in the development of effective AD treatments, given its multifactorial nature.[8,9] However, rational design of small molecules with therapeutic polypharmacology has always been a challenging task. Therefore, an efficient strategy that helps to identify novel chemical templates would be of great value in surmounting the paucity of effective disease-modifying agents in the pipeline of AD therapeutics.

Natural products have proven to be reliable resources in providing effective therapeutics for a variety of diseases. Recently, several small natural compounds with polypharmacological profiles have been shown to be of potential use in neurodegenerative disorders, among which curcumin (**1**, Figure 1)[10,11] and melatonin (**2**, Figure 1)[12,13] have been implicated in extensive studies as potential AD treatment agents. Compound **1**, a yellow spice and pigment isolated from the rhizome of *Curcuma longa*, has been tested in various AD models. The results have shown that **1** prevented Aβ-induced toxicity and lowered the level of Aβ in the brain, as well as the level of inflammatory cytokines and oxidative stress, thus demonstrating the potential of **1** as a promising candidate for treating human AD.[14] Melatonin (**2**, Figure 1), the major secretory product of the pineal gland, plays an essential role in the regulation of circadian rhythms.[15] In addition, **2** can be produced in various tissues and organs, and participates in diverse functions through both receptor-dependent and

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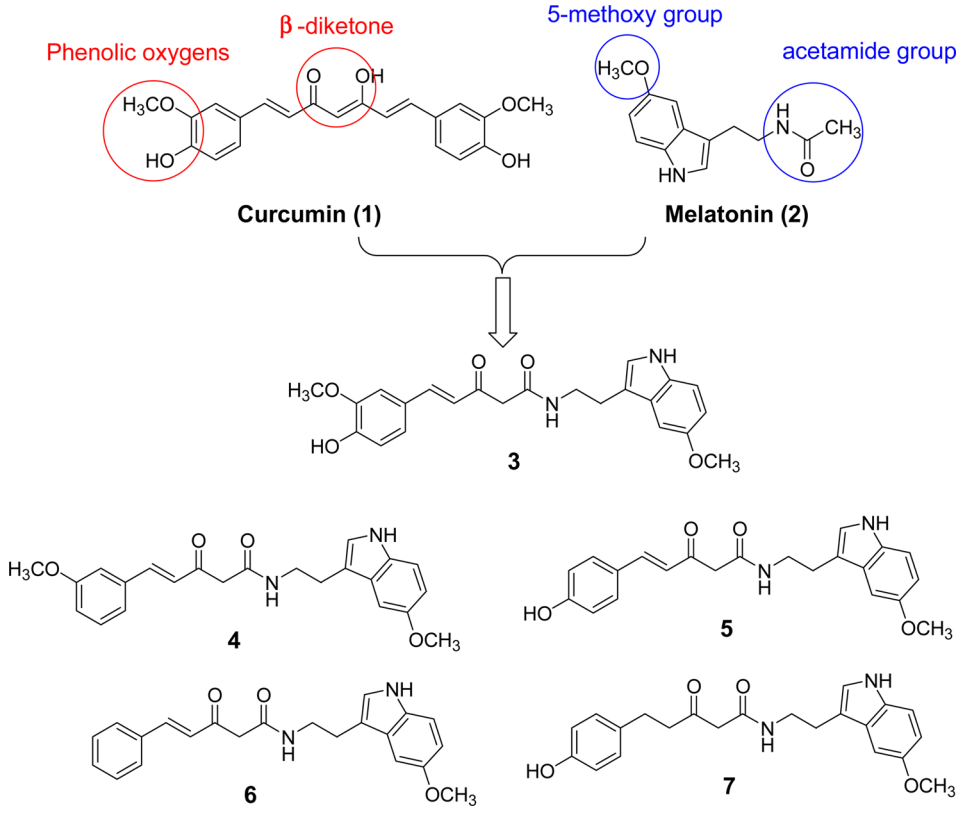
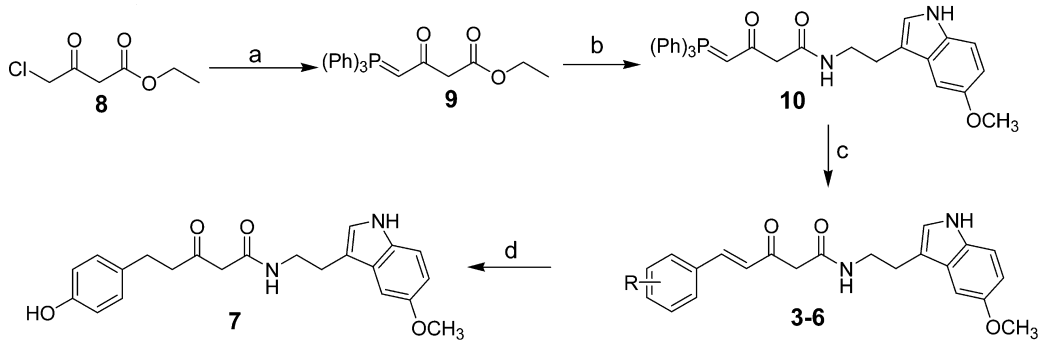


Figure 1. Chemical structures of curcumin (**1**), melatonin (**2**), and rationally designed hybrids **3**–**7**.

Scheme 1. Synthetic Route for the Preparation of 3–7[a]



[a]Reagents and conditions: (a) Ph_3P, benzene, Δ; (b) 5-methoxy-tryptamine, xylene, Δ; (c) NaH, aldehyde, DMPU/THF (1:1.2), or aldehyde, DMSO/H_2O (5:1), Δ; (d) H_2, Pd/C, MeOH.

independent ways, including free radical scavenging, immune response, and mood monitoring, among others.[13,16] Notably, circadian dysfunction and the reduction of **2** have been observed in AD, thus suggesting the potential of **2** in AD treatment.[17,18] Indeed, **2** has been tested as a potential treatment for AD (reviewed in ref 19). In transgenic AD mouse models, **2** has also been shown to improve cognition, and reduce Aβ deposition and neuroinflammation.[19,20] Clinical studies of **2** in AD patients also suggested beneficial effects, especially in sleep quality, reduced sundowning, and so forth.[21] However, more studies are needed to explore and investigate the usefulness of **2** as a treatment for AD.

Herein, we report the design and development of hybrids of **1** and **2** as an attractive strategy to develop effective neuroprotective compounds as potential AD-modifying agents. Recently, the "hybrid molecule" strategy has seen increased attention in drug design and development.[8] Given the demonstrated neuroprotective effects of **1** and **2** in various AD models and patients, we envisioned that a hybrid strategy would provide us with novel chemical scaffolds that either retain the functional natures of **1** and **2**, or provide new compounds with novel mechanisms of action, thus representing an attractive strategy to identify promising "hits" for further development.

RESULTS AND DISCUSSION

The desired hybrids must contain the structural features of **1** and **2** that are essential to their pharmacology properties. The phenolic oxygens and the β-diketone moiety of **1** have been demonstrated to be important for its antioxidant, anti-inflammatory, and metal chelating properties.[22] The 5-methoxy group and the acetamide moiety of **2** have been shown to be

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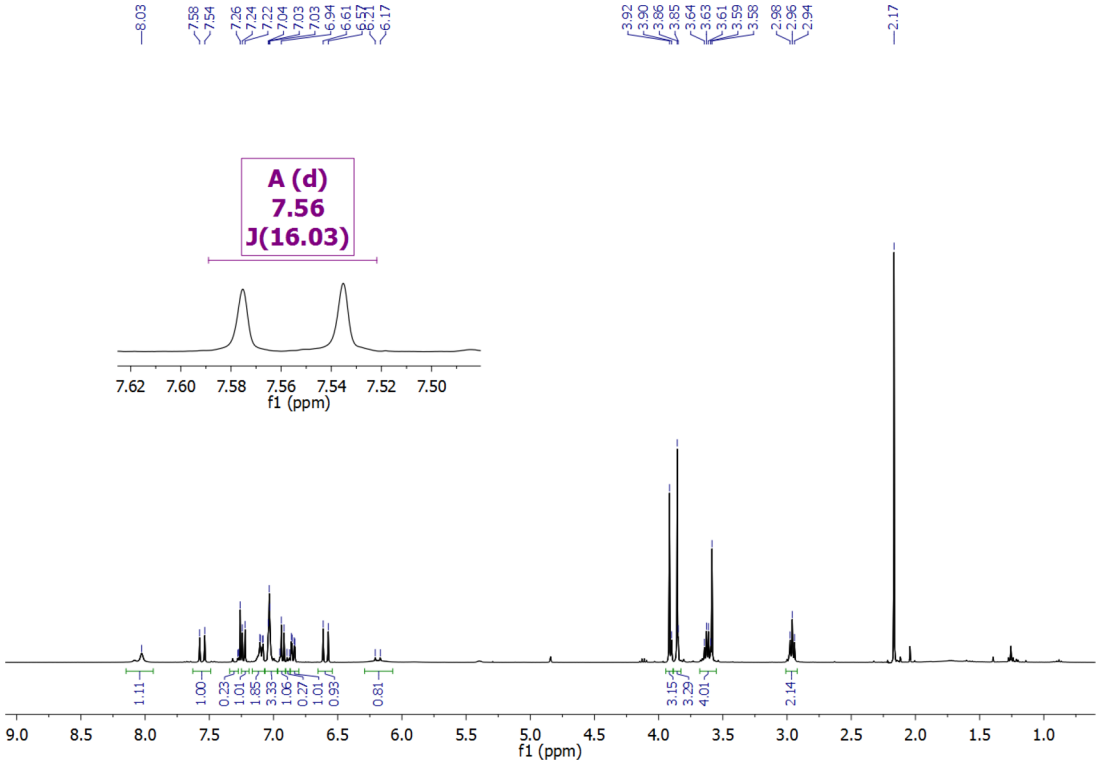

Figure 2. NMR spectrum of **3**. The coupling constant (J) of the two vicinal alkene protons is 16 Hz, indicating the trans-product of this structure.

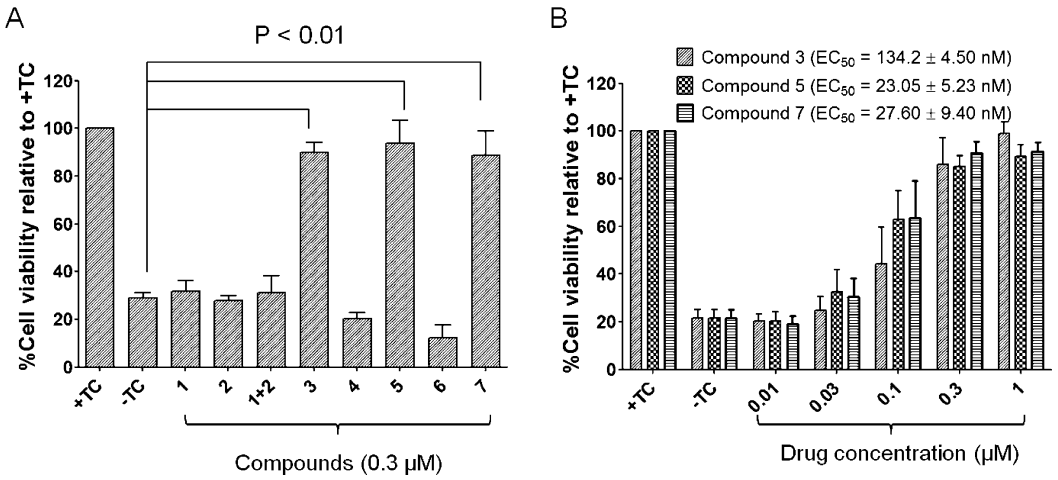

Figure 3. Neuroprotective effects of designed hybrids. (A) MC65 cells were treated with indicated compounds at 0.3 μM under +TC or −TC conditions for 72 h. Cell viability was assessed by MTT assay. Data were expressed as mean percentage viability (n = 3) with parallel +TC cultures set at 100% viability. Error bars represent SEM. (B) MC65 cells were treated with indicated compounds at indicated concentrations under −TC conditions for 72 h. Cell viability was assessed by MTT assay. Data were expressed as mean percentage viability (n = 3) with parallel +TC cultures set at 100% viability. Error bars represent SEM.

important for its antioxidant and free radical scavenging properties.[13] Therefore, in the newly designed hybrids, we intended to include all of these structural features. As shown in Figure 1, we initially designed hybrid **3** to incorporate the β-diketone of **1** and the acetamide moiety of **2** into a β-ketone amide moiety. We also used the indole moiety of **2** to replace one of the phenyl rings of **1**. In addition, several congeners of **3** (compounds **4**−**6**) were designed to evaluate the importance of the 4-OH and 3-CH_3O substitutions on the curcumin part of **3**, given the fact that structural modifications on the phenyl ring of **1** can significantly affect its biological activities.

The chemical synthesis of hybrids **3**−**6** was achieved following the procedures and conditions outlined in Scheme 1. Briefly, ethyl 4-chloroacetoacetate **8** was reacted with Ph_3P to give the ylide **9** in good yield. Condensation of **9** with 5-methoxy-tryptamine in xylene under refluxing conditions yielded **10**, which upon a Wittig reaction with the corresponding aldehyde in DMPU/THF in the presence of NaH, or in DMSO/H_2O under heating conditions finally afforded the designed hybrids **3**−**6**.[23] Interestingly, only the trans-product was obtained under these experimental conditions, which was demonstrated by the coupling constant (J)

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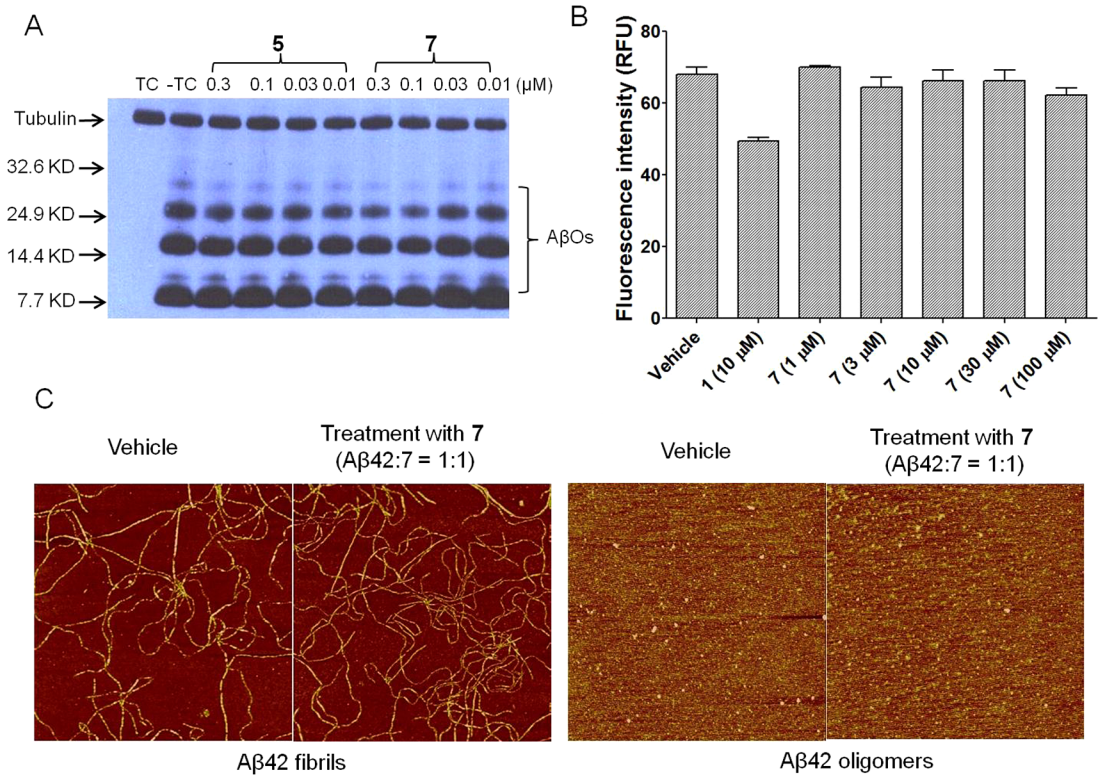

Figure 4. Mechanistic studies of **5** and **7**. (A) MC65 cells were treated with indicated compounds at indicated concentrations for 24 h immediately after the removal of TC. Lysates from cultures were analyzed by Western blotting using 6E10 antibody. The image represents the results from one of three independent experiments. (B) Aβ42 was added to solutions of **1** and **7** at indicated concentrations for 48 h. Thioflavin T (ThT) was then added, and fluorescence intensity was analyzed at 446 nm (excitation) and 490 nm (emission). Data were presented as a mean percentage of fluorescence intensity (n = 3). Error bars represent SEM. (C) Aβ42 fibrils and oligomers were incubated with a solution of **7** in a 1:1 ratio for 24 h. Aggregate morphology was visualized by AFM.

of the two vicinal alkene protons from **3** being 16.03 Hz (Figure 2).

After synthesizing these compounds, we evaluated their neuroprotective activities in MC65 cells, a well-established cellular AD model that is associated with Aβ- and oxidative stress-induced cellular toxicities, under tetracycline removal (−TC) conditions.[24] Initially, we tested at a concentration of 0.3 μM in order to establish active lead structures with reasonable potency. Compounds **1** and **2** alone and the combination of **1** and **2** were compared as controls. As shown in Figure 3A, no neuroprotection was observed in MC65 cells for **1** and **2** alone, as well as the combination of **1** and **2** under −TC conditions at this concentration. These results are consistent with our previously reported results of **1** in MC65 cells.[25,26] The combination of **1** and **2**, and **1** alone showed moderate neuroprotection at much higher concentrations (3 and 10 μM, data not shown). This also indicates that although **2** has been reported to have activity in other cellular models of neurodegenerative disorder, it might not be sufficient to protect MC65 cells under current testing conditions and concentrations. Notably, compound **3** significantly protected MC65 cells from −TC induced cell death (~61% increase in cell viability), which suggests that the combination of essential features of **1** and **2** can provide novel chemical scaffolds with new pharmacology. Removal of 4-OH from **3** as demonstrated by compound **4** led to a complete loss of neuroprotection in MC65 cells, while removal of 3-CH_3O did not affect its biological activity as compound **5** showed significant neuroprotection in MC65 cells. These results clearly indicate that the 4-OH group is essential to the neuroprotective activities of **3**. This notion is further supported by the results of the unsubstituted analogue **6**, which exhibited diminished protections of MC65 cells. To further evaluate the role of the double bond between the phenyl ring and the β-ketone, compound **7** was synthesized (Scheme 1) and evaluated. Notably, **7** exhibited significant and comparable protection of MC65 cells with **5** (Figure 3A), suggesting that the double bond and the conjugation system with the phenyl ring is not necessary to produce neuroprotection for these analogues. Interestingly, **7** can be recognized as the hybrid of **2** and raspberry ketone, another natural product, thus further supporting our hypothesis of the hybrid strategy. Further dose−response studies of **3**, **5**, and **7** established an EC_{50} of 134.2 ± 4.5, 23.05 ± 5.23, and 27.60 ± 9.40 nM, respectively, for their neuroprotection of MC65 cells (Figure 3B).

The promising and potent protective activities of this novel chemotype in MC65 cells strongly suggest that it may serve as a new template in developing more effective neuroprotectants for AD patients. Therefore, we decided to conduct further studies to obtain preliminary mechanistic data of this hybrid skeleton. This will help facilitate the design and evolution of next generation small molecules. Under -TC conditions, MC65 cells can produce intracellular AβOs that eventually lead to cell death. Therefore, we first evaluated the inhibitory effects of **5** and **7** on the production of AβOs in MC65 cells. As shown in Figure 4A, both **5** and **7** dose-dependently suppressed the production of AβOs, including tetramers, pentamers, and heptamers. However, the potency of **5** and **7** in suppressing

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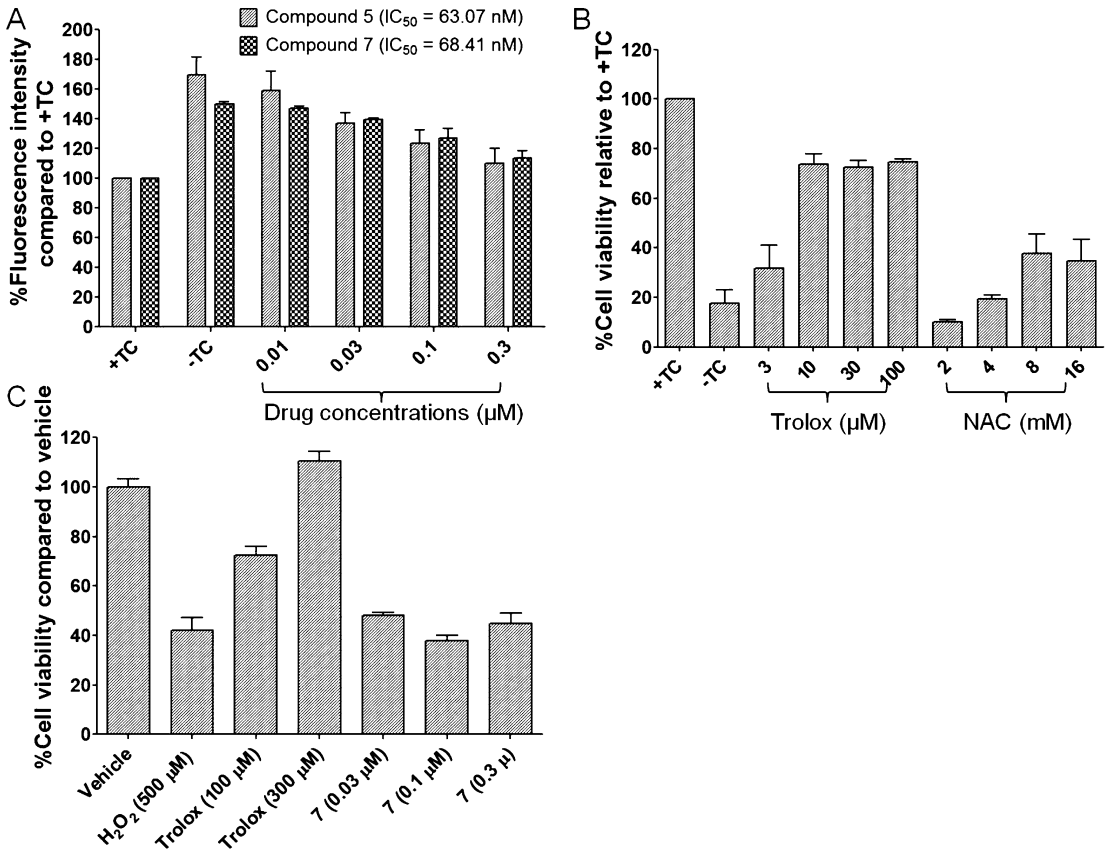

Figure 5. Antioxidative activity of **5** and **7**, and protective activities of known antioxidants. (A) MC65 cells were treated with **5** or **7** at indicated concentrations under −TC conditions for 48 h, then DCFH-DA (25 μM) was loaded and fluorescence intensity was analyzed at 485 nm (excitation) and 530 nm (emission). Data were presented as a mean percentage of fluorescence intensity (n = 3). Error bars represent SEM. (B) MC65 cells were treated with Trolox or NAC at indicated concentrations under −TC conditions for 72 h. Cell viability was assessed by MTT assay. Data were expressed as mean percentage viability (n = 3) with parallel +TC cultures set at 100% viability. Error bars represent SEM. (C) HT22 cells were treated with Trolox or **7** at indicated concentrations before addition of H_2O_2 (500 μM) and incubated for 24 h. Cell viability was assessed by MTT assay. Data were expressed as mean percentage viability (n = 3) with parallel H_2O_2-free cultures set at 100% viability. Error bars represent SEM.

AβOs is significantly lower than their potencies for protecting MC65 cells from −TC induced cytotoxicity (Figure 3B). This may suggest that the suppression of AβOs only contributes partially, if not at all, to their protection in MC65 cells and may not constitute the major mechanism of action. To further confirm effects on Aβ aggregation, we tested **7**'s ability to inhibit the formation of Aβ42 fibrils using the thioflavin T (ThT) assay. Compound **1**, known to inhibit Aβ fibrillization, was tested as a positive control. As shown in Figure 4B, **1** inhibited the formation of Aβ42 (25 μm) fibrils by 27.3% at 10 μM, consistent with reported results.[10] However, no significant inhibition was observed for **7** up to 100 μM, thus suggesting that **7** cannot bind to Aβ42 and inhibit its fibrillization. Atomic force microscopy (AFM) studies of Aβ42 aggregation also confirmed that **7** showed no inhibition on both fibrillization and oligomerization of Aβ42 under current assay conditions (Figure 4C).

Next, we checked whether **5** and **7** exhibit antioxidative effects in MC65 cells since oxidative stress has been suggested as one potential contributor to neurotoxicity upon the accumulation of intracellular AβOs. As shown in Figure 5A, both **5** and **7** dose-dependently suppressed intracellular oxidative stress, with an IC_{50} of ∼63 and ∼68 nM, respectively, being slightly less than their EC_{50} values from the neuroprotection assays. This may suggest that all of the upstream stimuli/signaling from the production of AβOs channels into oxidative stress that eventually leads to MC65 cell death. To further confirm this notion, we tested known antioxidants, *N*-acetylcysteine (NAC) and Trolox (6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid), for their protection in MC65 cells under the same assay conditions as **7**. Notably, like **7**, Trolox significantly protected cells from −TC induced cytotoxicity at concentrations as low as 10 μM. NAC only partially rescued cell viability at 8 and 16 mM concentrations (Figure 5B), which is consistent with our previously reported results.[25] Given the fact that Trolox and NAC have different antioxidative mechanisms, NAC being mainly a hydrogen peroxide scavenger and Trolox, a chain-breaking antioxidant, being particularly effective against lipid peroxidation,[27−29] this may suggest that ROS-induced lipid peroxidation is involved in the death of MC65 cells. Taken together, these results strongly support our notion that oxidative stress is the convergent event after the production of AβOs in MC65 cells that ultimately leads to cell death.

The manifested antioxidative effects of **5** and **7** in MC65 cells could be produced through different mecahnisms, for example, the inhibition of AβO production, the interactions of AβO with various partner proteins, or direct antioxidative effects. Therefore, we set out to investigate the possible mechanisms of **7**'s antioxidative effects. Since we have demonstrated that **7**

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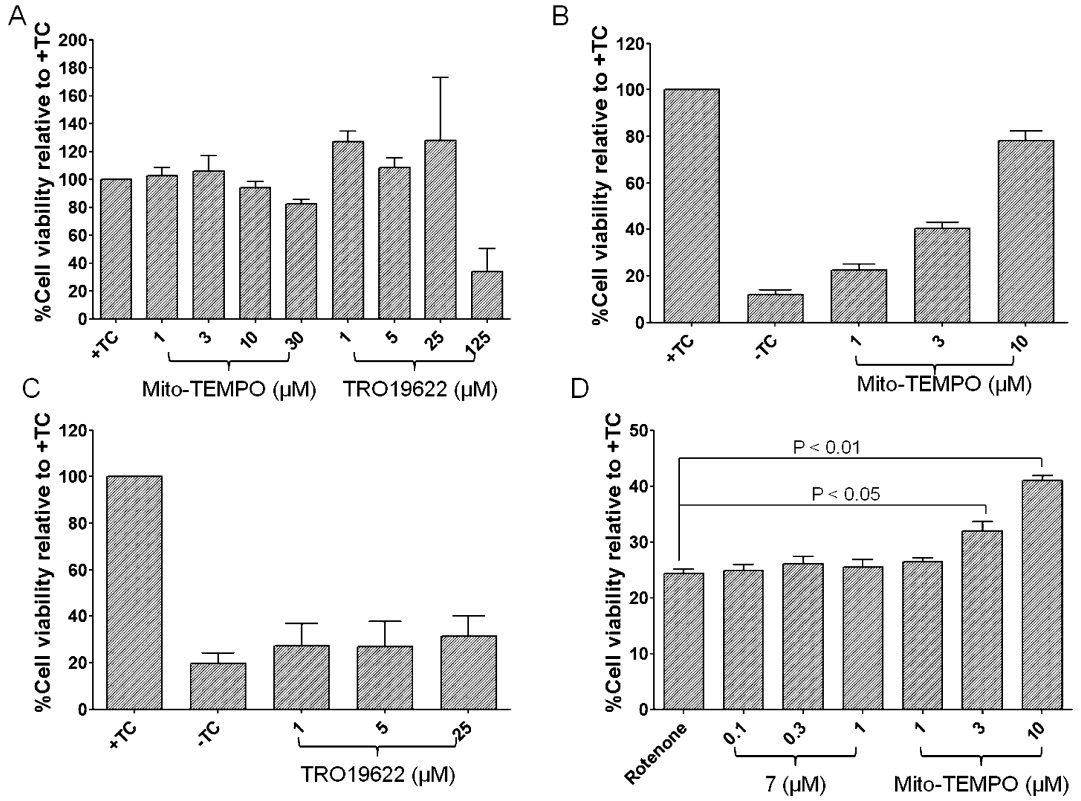

Figure 6. Effects on viability of TRO19622, Mito-TEMPO, and 7 in MC65 cells. (A) MC65 cells were treated with Mito-TEMPO or TRO19622 at indicated concentrations under normal growth conditions (+TC) for 72 h. Cell viability was assessed by MTT assay. Data were expressed as mean percentage viability (n = 3) with parallel +TC cultures set at 100% viability. Error bars represent SEM. (B) MC65 cells were treated with Mito-TEMPO at indicated concentrations under −TC conditions for 72 h. Cell viability was assessed by MTT assay. Data were expressed as mean percentage viability (n = 3) with parallel +TC cultures set at 100% viability. (C) MC65 cells were treated with TRO19622 at indicated concentrations under −TC conditions for 72 h. Cell viability was assessed by MTT assay. Data were expressed as mean percentage viability (n = 3) with parallel +TC cultures set at 100% viability. (D) MC65 cells were treated with 7 or Mito-TEMPO at indicated concentrations for 2 h before addition of rotenone for 48 h. Cell viability was assessed by MTT assay. Data were expressed as mean percentage viability (n = 3) with parallel +TC cultures set at 100% viability.

does not inhibit the aggregation of Aβ and inhibits the production of AβOs, but with a significantly lower potency compared to its neuroprotection potency in MC65 cells, we decided to test 7's ability to protect HT22 cells, a murine hippocampal line, from H_2O_2-induced cell toxicity, another widely used cellular antoxidant model.[30,31] The results from this assay will help rule out the possibility of 7's direct antioxidative effects. As shown in Figure 5C, H_2O_2 (500 μM) led to significant HT22 cell death (~58%). As expected, the known antioxidant Trolox dose-dependently protected cells from H_2O_2-induced cytotoxicity with full rescue at 300 μM. However, no protection was observed for 7 at up to 0.3 μM under the same experimental conditions. Taken together, the results suggest that the effects of 7 observed in MC65 cells are not through direct antioxidation, thus indicating that 7 may be functioning somewhere between the production of AβOs and the accumulation of ROS, for example, the interactions of AβO with partner proteins.

Since mitochondria are the main sites to generate intracellular ROS,[32,33] we further investigated whether 7 functions in mitochondria to exhibit its antioxidant and neuroprotective activities. To that end, we first tested the neuroprotective effects of TRO19622, a mitochondrial permeability transition pore (mPTP) inhibitor,[34] and Mito-TEMPO, a known mitochondrial ROS (mitoROS) specific scavenger,[35] on MC65 cells from −TC induced cytotoxicity since mPTP has been reported to be associated with mitochondrial ROS production,[36] and 7 has shown antioxidant effects in MC65 cells. Initially, we tested these two compounds under normal growth conditions (+TC) in MC65 cells to identify concentrations with no cytotoxic effects, thus ruling out any potential biased interpretation of the following assays. As shown in Figure 6A, Mito-TEMPO did not show toxic effects up to 10 μM and TRO19622 did not show cytotoxicity up to 25 μM. Therefore, these two concentrations were chosen as maxima for their respective compounds in the following tests. As shown in Figure 6B, Mito-TEMPO dose-dependently protected MC65 cells from −TC induced cytotoxicity, while TRO19622 did not show significant protection up to 25 μM (Figure 6C). Combining these with the results of 7's antioxidant and neuroprotective effects, this may indicate that AβOs, produced upon TC removal, interact with certain mitochondrial membrane proteins to generate specific mitoROS in a mPTP formation and opening-independent manner. To further confirm this notion, we employed rotenone, a neurotoxin that has been demonstrated to inhibit mitochondrial complex I and is linked to mitoROS production, in MC65 cells to study the protective effects of 7 and Mito-TEMPO. As shown in Figure 6D, rotenone significantly induced cell death of MC65 cells (75.6%) at 10 μM. Mito-TEMPO significantly protected MC65 cells from rotenone-induced cytotoxicity at 3 and 10 μM in a dose-dependent

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manner, while 7 did not show protection up to 1 μM, concentrations known to protect MC65 cells from −TC induced cytotoxicity. Taken together, these results suggest that, upon production, AβOs enter or interact with the mitochondrial membrane to produce mitoROS that lead to the death of MC65 cells, and 7 blocks this interaction of AβOs with mitochondria, thus ultimately leading to its antioxidant and neuprotective activities as demonstrated by the aforementioned assays. Given the fact that AβOs have been shown to induce tau hyperphosphorylation, neurofibrillary tangle formation, synaptic alteration, and neurodegeneration,[5,37−40] the ability of 7 to block the interactions of AβOs with partner proteins may suggest the potential of this chemotype as a new template to develop more effective AD-modifying agents. Further studies are warranted to elucidate the mechanisms of this compound.

In addition to activity, it is essential to establish whether these compounds are able to cross the blood brain barrier (BBB) as they are destined to act within the central nervous system (CNS). Therefore, we evaluated the potential brain penetration of **3**, **5**, and 7 using the optimized parallel artificial membrane permeability-BBB (PAMPA-BBB) passive diffusion model, a well-established and widely used in vitro BBB model for molecules with limited water-solubility.[41,42] The in vitro permeability (P_e) values of **3**, **5**, and 7 through a lipid extract of porcine brain were determined by using a mixture of PBS and ethanol in the ratio of 70:30. In the same assay, 10 commercial drugs with known CNS penetration were also tested as positive controls, and their experimental values were compared to reported values (Table 1). The results gave a good linear correlation with P_e (exp) = 1.603P_e (bibl) + 4.860 (R^2 = 0.876). From this equation and following the pattern established in the literature for BBB permeability prediction, we expect that compounds with P_e < 8.1 × 10^{-6} cm s^{-1} will have low BBB permeability by passive diffusion. As shown in Table 1, all three compounds showed P_e values under this limit with 7 giving the highest value, so we suspect that these analogues might experience some difficulty in reaching the brain by passive diffusion. However, considering the predictive nature of this assay and the existence of influx transporters in the BBB, for example, caffeine is not able to cross BBB by passive diffusion, but it reaches brain through a nucleoside transport system, we could not rule out the possibility that our compounds can reach brain tissues by a carrier-mediated penetration mechanism. Therefore, we decided to test the BBB penetration in intact mice. Both compounds **5** and 7 exhibited comparable neuroprotection potencies in the MC65 cell model, and the preliminary mechanistic studies demonstrated that they share the same mode of actions. Therefore, based on the observed solubility and stability from our in vitro tests and also considering the cost of further in vivo studies using transgenic AD mice, we only selected 7 for further BBB penetration studies in mice.

Table 1. Permeability (P_e = 10^{-6} cm s^{-1})[a] in the PAMPA-BBB Assay for Commercial Drugs (used for experimental validation) and Compounds 3, 5, and 7 with Their Predictive Penetration in the CNS

commercial drugs	bibl[b]	P_e (exp.)	compd	P_e (exp)	prediction
testosterone	17.0	31.4 ± 2.4	7	7.9 ± 0.6	CNS −
verapamil	16.0	31.2 ± 2.1	5	5.9 ± 0.4	CNS −
imipramine	13.0	18.6 ± 1.8	3	6.5 ± 0.6	CNS −
desipramine	12.0	28.5 ± 0.5			
promazine	8.8	25.1 ± 1.4			
corticosterone	5.1	10.9 ± 1.0			
piroxicam	2.5	6.0 ± 0.3			
hydrocortisone	1.9	10.0 ± 0.6			
caffeine	1.3	8.2 ± 0.6			
ofloxacin	0.8	5.3 ± 0.3			

[a]PBS/EtOH (70:30). Data are the mean ± SD of three independent experiments. [b]Taken from ref 41.

Given the consideration that management of AD with medications would be a long-term care process for patients and oral administration would significantly improve patient compliance, we tested 7 for its BBB permeability in male CD-1 mice (n = 6) by oral administration at a dose of 50 mg/kg. To accurately quantify the amount of 7 that is delivered into brain tissue and rule out the possibility of biased interpretation from vascular trapping, we perfused the mice to wash out the vascular blood completely prior to collecting brain tissues. After oral administration, plasma samples were collected at 0.25, 0.5, 1, and 24 h, and brain samples were collected at 1 and 24 h. Collected samples were analyzed by LC-MS/MS, and the results are shown in Table 2. Compound 7 exhibited a quick absorption profile as the plasma concentration reached 773 ± 309.86 nM (n = 6) 15 min after oral administration and was only slightly increased after 1 h. The plasma and brain concentrations of 7 after 1 h were 883.12 ± 350.36 and 555.40 ± 188.43 nM, respectively. This clearly indicates that 7 quickly and efficiently reached brain tissue after oral ingestion, thus confirming its BBB permeability. After 24 h, the plasma and brain concentrations dropped to 30.53 ± 11.96 and 47.41 ± 11.19 nM, respectively. It is important to note that the brain concentration of 7 at this time point still remains above the neuroprotective EC_{50} of 7 in MC65 cells (27.60 ± 9.60 nM), suggesting that a once daily regimen should provide a sufficient amount of 7 in the brain tissue to be therapeutically effective.

In summary, hybrid compounds of curcumin (**1**) and melatonin (**2**) were designed and synthesized as potential neuroprotectants for AD. Initial biological characterization of **3** from in vitro assays established that the hybrid strategy is a viable approach in providing novel chemotypes with novel pharmacology. Further modifications identified 7 as a lead compound with potent neuroprotections in MC65 cells. Preliminary mechanistic studies suggested that antioxidative effects might be the major mechanism leading to their neuroprotection, and it is likely that the manifested antoxidative effects of 7 are through interference of the interactions of AβOs with the mitochondria in MC65 cells. Furthermore, 7 has been shown to penetrate the BBB efficiently after oral adminsitration in intact mice, thus confirming that it is orally bioavailable and

Table 2. Plasma and Brain Concentrations (nM) of 7 after Oral Administration at 50 mg/kg Dose in CD-6 Mice (n = 6)

	15 min	30 min	1 h	24 h
plasma	773.80 ± 309.86	794.72 ± 301.34	883.12 ± 350.36	30.53 ± 11.96
brain			555.40 ± 188.44	47.41 ± 11.19

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therapeutically relevant concentrations are attainable in CNS. These results strongly encourage further studies and optimization of **7** in development of more potent analogues. These findings also support the hybridization strategy as a novel design approach to provide effective disease-modifying agents for AD.

METHODS

Biological Assays. Aβ42 was obtained from American Peptide, Inc. (Sunnyvale, CA). 6E10 antibody was obtained from Signet (Dedham, MA). MC65 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT), 1% penicillin/streptomycin (P/S) (Invitrogen), 1 μg/mL tetracycline (TC) (Sigma-Aldrich, St. Louis, MO), and 0.2 mg/mL G418 (Invitrogen). HT22 mouse hippocampal cells were cultured in DMEM supplemented with 10% FBS and 1% P/S. All cells were maintained at 37 °C in a fully humidified atmosphere containing 5% CO_2. CD-1 male mice were purchased from Harlan Laboratories (Frederich, MD). All experiments involving animals were carried out in strict accordance with the recommendations in the Guidelines and Regulations of Institutional Animal Care and Use Committee (IACUC) of the Virginia Commonwealth University (VCU). The protocol was approved by the Committee on the Ethics of Animal Experiments of VCU (IACUC Number: AD20114).

Neuroprotection Assay in MC65 Cells. MC65 cells were washed twice with PBS, resuspended in Opti-MEM, and seeded in 96-well plates (4×10^4 cells/well). Indicated compounds were then added, and cells were incubated at 37 °C under +TC or −TC conditions for 72 h. Then, 10 μL of MTT (5 mg/mL in PBS) was added and the cells were incubated for another 4 h. Cell medium was then removed, and the remaining formazan crystals produced by the cellular reduction of MTT were dissolved in DMSO. Absorbance at 570 nm was immediately recorded using a FlexStation 3 plate reader (Molecular Devices, CA).

ROS Production Assay in MC65 Cells. MC65 cells were washed twice with PBS, resuspended in Opti-MEM, and seeded in 6-well plates (8×10^5 cells/well). Indicated compounds were then added, and cells were incubated at 37 °C under +TC and −TC conditions for 48 h. Cells were harvested, washed twice with cold PBS, suspended in PBS, and then incubated with DCFH-DA (25 μM) in dark for 1 h. Fluorescence was analyzed by flow cytometry using a Millipore Guava easyCyte flow cytometer.

Western Blot Assay. MC65 Cells (4×10^5 cells/mL) were treated with indicated compounds for 30 h and then were lysed by sonication in a Tricine buffer solution and boiled for 5 min. Protein samples were collected from the supernatant after centrifugation of the samples at 12 800*g* for 5 min and then quantified using the Bradford method. Equal amounts of protein (20.0 μg) were separated by SDS-PAGE on a gel (Bio-Rad) and transferred onto a PVDF membrane (Bio-Rad). The blots were blocked with 5% milk in TBS-Tween 20 (0.1%) solution at room temperature for 1 h and then probed with the 6E10 antibody overnight at 4 °C. The blots were washed twice in TBS-Tween 20 for 15 min, and then incubated with a 1:1000 dilution of horseradish peroxidase-conjugated secondary antibody in a 5% milk/PBS-Tween 20 solution at room temperature for 1 h. After washing twice in TBS-Tween 20 for 15 min, the proteins were visualized by using a Western blot chemiluminescence reagent (Thermo Fischer Scientific, Waltham, MA). The blots were also probed with antibodies against α-tubulin to ensure equal loading of proteins.

Thioflavin T Assay. Briefly, 1 μL of each compound solution in DMSO (0.01−100 μM) was added to corresponding wells in a 96-well plate. Each concentration was prepared in independent triplicates, and a solvent control was included. To each well, 9 μL of 25 μM Aβ42 in PBS (pH 7.4) was added, and then plates were incubated in dark at room temperature for 48 h. Next, 200 μL of a 5 μM ThT in 50 mM glycine solution (pH 8.0) was added to each well. Fluorescence was immediately recorded using a FlexStation 3 plate reader (Molecular Devices, CA) at an excitation wavelength of 446 nm and an emission wavelength of 490 nm.

AFM Analysis of Aβ1−42 Fibril and Oligomer Formation. Aβ42 oligomers and fibrils were prepared based on reported procedures.[43] Indicated compounds were incubated with Aβ42 at a 1:1 ratio for both conditions for 24 h. Samples were loaded on mica, washed extensively with water, and dried overnight at room temperature before AFM analysis. The morphology of the Aβ42 aggregates was assessed using an atomic force microscope (Dimension Icon, Bruker) operating in tapping mode in air. The scan rate was varied between 1 and 0.5 Hz depending on the tracking quality. The silicon tips (Bruker mpp2100-100) have a sharpness of less than 5 nm and a force constant between 3 and 5 N/m along with a resonant frequency rated between 60 and 90 Hz. All images were taken with 512 points per line, with a 1:1 ratio. Images were processed using Nanoscope analysis software version 1.20 and Image-J (from the National Institutes of Health).

Hydrogen Peroxide Toxicity Assay in HT22 Cells. HT22 cells were seeded in 96-well plates (4×10^3 cells/well) in growth medium and incubated for 24 h at 37 °C. The medium was removed, the compounds were added at the indicated concentrations in fresh growth medium, and the cells were incubated for another 1 h. H_2O_2 was then added at a final concentration of 500 μM, and the plates were then incubated for 24 h. Cell viability was assessed by MTT assay as previously described. Values were expressed as a percentage relative to the negative (H_2O_2-free) control.

Rotenone Protection Assay in MC65 Cells. MC65 cells were seeded in 96-well plates (4×10^4 cells/well) in growth medium and incubated for 24 h at 37 °C. The medium was removed, the compounds were added at the indicated concentrations in fresh growth medium, and the cells were incubated for another 2 h. Rotenone was then added at a final concentration of 10 μM, and the plates were then incubated for 48 h. Cell viability was assessed by MTT assay.

In Vivo BBB penetration experiment. Briefly, 10 week old CD-1 male mice were purchased from Harlan Laboratories (Frederich, MD). Compound **9** was diluted to a concentration of 15 mg/mL in a solution with 2% DMSO and 10% Cremophor in PBS, and was administered via oral gavage at a final dosage of 50 mg/kg. Two groups of mice (n = 6/group) were used to determine the plasma and brain concentrations of the compound at various time points. Following administration of anesthetic (sodium pentobarbital 150 mg/kg, Sigma-Aldrich, Saint Louis, MO), blood samples were collected from the inferior vena cava to prepare plasma. Afterward, the right atrium was removed to allow exsanguination, and the left ventricular apex was cannulated with a 24 G needle and perfused with 30 mL of warm (37 °C) heparinized normal saline solution to enable perfusion of all the organs and complete blood washout. The perfused brains were then collected, rapidly washed in normal saline, blot-dried, and frozen in liquid nitrogen. Brain samples were then analyzed by the LC-MS/MS.

ASSOCIATED CONTENT

Supporting Information

Details of chemical synthesis and LC-MS/MS analysis of brain and plasma samples. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

#J.E.C. and K.L. contributed equally to this manuscript. Chemical synthesis, MC65 neuroprotection, antioxidant, and HPLC studies were completed by K.L. and J.E.C.; Western blot, HT22 cell studies, and ThT assays were completed by K.L.; Mito-TEMPO and TRO19622 studies in MC65 cells

were completed by X.Y.; AFM studies were performed by T.S. and D.Y.; PAMPA-BBB studies were completed by M.E. and M.I.R.-F.; LC-MS/MS studies were completed by K.L. and M.S.H.; BBB studies in mice were performed by S.T.; Experiment design, data analysis, writing, and editing were completed by 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; AFM, atomic force microscopy; BBB, blood-brain barrier; CNS, central nervous system; DCFH-DA, dichlorofluorescein diacetate; DMPU, *N*,*N*′-dimethylpropyleneurea; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; ESI, electrospray ionization; FBS, fetal bovine serum; HPLC, high performance liquid chromatography; LC, liquid chromatography; mitoROS, mitochondrial reactive oxygen species; mPTP, mitochondrial permeability transition pore; MS, mass spectrometry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, *N*-acetylcysteine; PAMPA, parallel artificial membrane permeability; PBS, phosphate buffered saline; PMA, phosphomolybdic acid; ROS, reactive oxygen species; SAR, structure−activity relationship; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEM, standard error of mean; SRM, selected reaction monitoring; TBS, Tris buffered saline; TC, tetracycline; TFA, trifluoroacetic acid; THF, tetrahydrofuran; ThT, thioflavin T; TLC, thin-layer chromatography; TMS, tetramethylsilane

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