Synthesis and Bioactivity Evaluation of a Novel 1,2,4-Oxadiazole Derivative in vitro and in 3×Tg Mice

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Aim: Alzheimer’s disease (AD) is the most common neurodegenerative disease whose patients suffered from cognitive impairments. In our study, a novel 1,2,4-Oxadiazole derivative wyc-7-20 was synthesized, which showed low cytotoxicity and potent neuroprotective effect at the cellular level. Improved cognitive impairments, β-amyloid (Aβ) clearance, and tau pathological phenotypes were detected in transgenic animal models after wyc-7-20 treatment. Reversed expressions in AD-related genes were also detected. The results demonstrated wyc-7-20 was potent in AD therapy.

Purpose: The pathological complexity of AD increased difficulties in medical research. To explore a new potential medical treatment for AD, a novel 1,2,4-Oxadiazole derivative (wyc-7-20) was designed, synthesized to explore the application in this study.

Materials and Methods: Human neuroblastoma (SH-SY5Y) cells and human hepatocellular carcinoma (HepG2) cells were used to detect median lethal dose (LD50). H2O2 and Aβ1–42 oligomers (AβOs) were respectively, added into SH-SY5Y cells to detect anti-ROS (reactive oxygen species) and anti-AβOs effects of wyc-7-20. 3×Tg mice were administered with wyc-7-20, and then Y maze test and Morris water maze (MWM) test were applied to detect cognitive improvements. Brain tissue samples were subsequently collected and analyzed using different techniques.

Results: wyc-7-20 showed low cytotoxicity and potent neuroprotective effect at the cellular level. Improved cognitive impairments, Aβ clearance, and tau pathological phenotypes were detected in transgenic animal models after wyc-7-20 treatment. Reversed expressions in AD-related genes were also detected.

Conclusion: wyc-7-20 was potent in AD therapy.

Keywords: 1,2,4-oxadiazole, Alzheimer’s disease, animal model, neuroprotective effect

Introduction
As the leading cause of dementia, Alzheimer’s disease (AD) is a neurodegenerative disease and affects tens of millions of people globally.1,2 Unfortunately, the number of AD sufferers has continuously been increasing by a fold every 2 decades.3 AD is a multi-risk factor disease, which is associated with a variety of acquired factors and genetic factors.4 Metabolic syndrome such as obesity and diabetes raised the risks for AD.5,6 The biological processes of metabolism, inflammation, synapse formation, APP processing, and cytoskeletal alterations are involved in the pathogenesis and pathological development of AD.5–9

Although advances in AD pathophysiology have been made in recent years, the disease’s heterogeneity and complexity have surely increased difficulties in medical treatment research.1 Bioactive compounds containing 1,2,4-Oxadiazole attracted growing attention in recent years. In the medicinal chemistry, 1,2,4-oxadiazole was usually considered as bio-isosteres of carbamates, amides and esters.10 As a five-membered heterocycle, the advantage of
1,2,4-oxadiazole lied in structural characteristics, including lone pair electrons and aromaticity. Another important contribution was chemical stability, both in aqueous solution and in organic phase. The effects of 1,2,4-oxadiazole derivatives on anti-bacterial infection, anti-inflammation, anti-tumor, anti-virus and anti-oxidant activity have been investigated. Inspired by diverse pharmacological effects and the privileged scaffold, we attempted to explore the application of 1,2,4-oxadiazole derivatives in the treatment of AD. In this work, we found a novel 1,2,4-oxadiazole derivative (wyc-7-20) with low toxicity on SH-SY5Y and HepG2 cells and improvement of the AD phenotypes of 3×Tg mice.

**Materials and Methods**

**Chemistry**

All chemicals were commercially available without further purification. $^1$H NMR spectra were recorded on the Varian Mercury 400 spectrometer (Figure S1). An Agilent Technologies LC/MSD TOF instrument was used to record high-resolution mass spectra.

Compounds 2–4 were synthesized by the reported method. Compound 4 (1 mmol) and 1-[(4-methylphenyl) carbonyl] piperazine (1 mmol) were added to a solution of acid derivatives (1.2 mmol) in toluene (10 mL). The mixture was refluxed for 8 h. After evaporation, the crude product was purified by flash column chromatography to afford wyc-7-20. Yield: 88%; $^1$H NMR (400 MHz, CDCl$_3$) δ 9.33 (s, 1H), 8.76 (d, $J$ = 3.6 Hz, 1H), 8.38 (dt, $J$ = 8.0, 1.9 Hz, 1H), 7.48–7.42 (m, 1H), 7.32–7.28 (m, 2H), 7.23–7.18 (m, 2H), 4.00 (s, 2H), 3.70 (d, $J$ = 98.6 Hz, 4H), 2.70 (s, 4H), 2.37 (s, 3H); HR-ESI-MS: $m/z$ calcd for C$_{20}$H$_{22}$N$_5$O$_2$ $^+ 364.1773$ ([M+H]$^+$), found 364.1776.
Cell Culture Studies

All the cell lines were purchased from Chinese National Biomedical Experimental Cell resource Bank (BMCR). Cells were cultured in a previously described method. Briefly, human neuroblastoma (SH-SY5Y) cells and human hepatocellular carcinoma (HepG2) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, 11965065), supplemented with 15% fetal bovine serum (FBS, EVERY GREEN, 11,011–8611, CA), 2 mM l-glutamine and 0.10 mg/mL antibiotic penicillin-streptomycin solution (Solarbio, T1320, CA). All of the cells were cultured in a humidified atmosphere of 5% CO₂ at 37°C.

Cell Viability Assays

For the median lethal dose (LD50) assay, SH-SY5Y cells and HepG2 cells were seeded in 96-well plates, respectively, at a density of $1.2 \times 10^4$/well and cultured for 24 h. A series of concentrations of wyc-7-20 and evodiamine (0.000010 μg/mL to 300.0 μg/mL) were then added into 96-well plates, respectively, and cultured continually for 24h. The LD50 of wyc-7-20 on SH-SY5Y and HepG2 cells were detected by cell viability with Cell Counting Kit-8 (CCK-8, Beyotime, C0039) following the modified instruction. Briefly, each well received 10 μL CCK-8 buffer and was incubated at 37°C for 2 h. The absorbance at 450nm (OD) was then read by a microplate reader (Thermo Fisher, 51119200). The percentage of cell viability was calculated by normalizing the value of the vehicle control.

For the anti-ROS assay, SH-SY5Y cells were seeded in 96-well plates at a density of $1.2 \times 10^4$/well and cultured for 24 h. The cells were treated with H₂O₂ alone (1300 μM), H₂O₂ with evodiamine or wyc-7-20 at indicated concentrations for 24 h. The cell viability was detected with CCK-8, and the percentage of cell viability was calculated by normalizing the value of the H₂O₂ control.

Aβ Cytotoxicity Assay

The Aβ₁₋₄₂ oligomers (AβOs) were obtained from Chinapeptides (04010011526). The protective effect of wyc-7-20 on Aβ cytotoxicity was detected by the real-time cell analyzer (ACEA Biosciences, xCELLigence RTCA DP, USA) as the reported. Briefly, SH-SY5Y cells were seeded in E-plates (ACEA Biosciences, 0030060890, USA) at a density of $6 \times 10^3$/well (150 μL) and cultured for 3 h to allow the cell attachment. The culture medium was removed and the fresh medium with AβOs alone (15 μM) or the AβOs with wyc-7-20 (0.1 μg/mL) was added into the plates. The plate was placed on the cell function analyzer in a CO₂ incubator and cultured at 37°C for 69 h. The real-time cell viability and cell number were indicated by the cell indexes (CI), which were estimated by a software of the Live Cell Station.

Animals and Groups

The wild-type C57/BL6J and 3×Tg AD model mice expressing APP Swedish, PSEN1 M146V and MAPT P301L were bred in our AAALAC-accredited facility. The 3×Tg mice were originally obtained from Jackson Lab and transferred to C57/BL6J background by crossing with wild-type C57/BL6J for 12 generations. The 3×Tg mice developed AD phenotypes from 4 months old, such as cognitive deficits, Aβ deposits, gliosis, and Tau phosphorylation. The WT and 3×Tg mice at 10 months old were randomly allocated into WT group (n = 6), 3×Tg group (n = 6), and wyc-7-20 group (n = 6). The wyc-7-20 was dissolved in 20% polyethyleneglycol (PEG-200) and then diluted in normal saline (NS) to 0.16 mg/mL. The mice were treated with vehicle or wyc-7-20 at 200 μg/kg via intraperitoneal injection (i.p) every 2 days for a month.

Y Maze Test

The working memory of mice was detected by the Y maze apparatus (30 × 5 × 15cm) and the 3 arms were conveniently named as A, B, and C. Protocol of the Y maze test was modified from reported methods. The mice were released at the end of one arm and freely explore for 5 min. When all of the four limbs of a mouse within one arm were considered to finish an arm entry and the entry number was recorded (Noldus, Ethovision XT). A sequence of mice into 3 different arms was considered a spontaneous alternation (A-B-C or A-C-B). Alternative percentage (%) = alternative number/(total entry-2) × 100. After each test, the apparatus was cleaned with 75% ethanol and dried thoroughly.
Morris Water Maze (MWM) Tests
Protocol of MWM test was modified from reported methods. Backed to the center of the maze, mice were released from the center of one quadrant without the platform. Mice undertook training for 5 consecutive days and started from different releasing quadrants twice daily. Latencies to find the invisible platform were recorded. If the mouse failed to find the platform within the 60 s, latency was recorded as 60s and the mouse was guided to the platform and stayed there for 5 s. On day 6, the platform was removed. Mice started from the southwest quadrant to freely explore for 60 s. Crossing times to the previous platform region (northeast quadrant) occupancy were recorded. A video tracking system (Noldus, Ethovision XT) was used for record and analysis.

Western Blot
Brain tissues were homogenized in RIPA (Beyotime, P0013b) containing protease and phosphatase inhibitor cocktail (Thermo, 78444). Protocol of Western Blot was modified from reported methods. The samples were separated on a 10% polyacrylamide gradient SDS-PAGE gel (Beyotime, P0012A) and transferred to 0.45μm nitrocellulose blotting membrane (Millipore, Immobilon NC). Membranes were blocked with 5% bovine serum albumin (GENVIEW, FA016-100G) in TBST buffer. Antibodies against phosphorylated Tau at Ser202/Thr205 (p-Tau, Thermo, MN1020, 1:500) and anti-Tau5 (Millipore, 577801, 1:500) were respectively incubated with the NC membrane at 4°C overnight. Anti-GAPDH (Abcam, ab201822) was used as a control. The membranes were subsequently incubated with peroxidase-linked anti-mouse IgG antibody (Zhongshan Golden Bridge, ZB2305) and visualized by ECL (Santa Cruz, sc-2048). Proteins were visualized by the chemiluminescent detection system (Bio-Rad Laboratories, Inc. version 3.0) and density was analyzed with Image J.

Immunofluorescence
The left hemispheres of mice brains were fixed in 4% formalin solution overnight and then transferred to 30% saccharose solution. The 14 μm frozen sections were prepared and maintained at −20°C. The sections were stained with anti-β-amyloid antibody (6E10, Biolegend, 803002) overnight at 4°C. The FITC-labeled goat anti-mouse IgG (Zhongshan Golden Bridge, ZB2305) was used to report the first antibody. Finally, slices were sealed by mounting medium with DAPI (Zhongshan Golden Bridge, ZLI-9557). Slices were scanned with the fluorescencescanner (3D HISTECH, pannoramic 250) and the FITC marked plaques and cells were subsequently analyzed with Image J.

RT-qPCR
Total RNA of brain tissues was isolated using TriQuick Reagent (SolarBio, R1100) according to the previous protocol. The cDNA was transcribed using a kit (TransGen Biotech, AU341-02). Expression of 6 AD-related genes were detected by RT-qPCR, including cytochrome P450 family 2 subfamily E member 1 (Cyp2e1), fatty acid-binding protein 3 (Fabp3), fatty acid-binding protein 7(Fabp7), S100 calcium-binding protein B (S100b), glutamate ionotropic receptor alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate type subunit 1 (Ampar1), discs large membrane-associated guanylate kinase scaffold protein 4 (Psd95). The 6 pairs of primers were summarized in Table 1. The RT-qPCR was performed using a qPCR kit (TransGen Biotech, AQ602) on Quant Studio3 system (Thermo, ABI 7500). Fold changes of PCR products were determined by comparative cycle times after endogenous reference gene (glyceraldehyde-3-phosphate dehydrogenase, Gapdh) normalization.

Statistical Analysis
Data were analyzed using GraphPad Prism (Version 8.0, GraphPad Software, La Jolla, CA, USA) and presented descriptively as the mean ± standard error of the mean (SEM). Differences between the two groups were assessed by an unpaired t-test. P<0.05 was considered statistically significant.

Results
Chemical Synthesis of Wyc-7-20
The synthesis of wyc-7-20 was started from 3-cyanopyridine (1), which reacted with hydroxylamine hydrochloride to give hydroxymididine 2. Compound 3 was subsequently afforded according to the treatment of 2 with chloroacetyl.
chloride. The intermediate 4 was obtained after cyclization in toluene. Finally, the intermediate 4 was reacted with 1-[(4-methylphenyl) carbonyl] piperazine to provide wyc-7-20 in good yield. Its structure was confirmed by 1H-NMR and HR-MS.

Wyc-7-20 Exists Lower Cytotoxicity on SH-SY5Y and HepG2 Cells

The cytotoxicity of wyc-7-20 was firstly analyzed on SH-SY5Y cells and HepG2 cells, which were commonly used for neurotoxicology and hepatotoxicity in vitro analysis. The LD50 of wyc-7-20 was compared with evodiamine, which improved AD symptoms but with side effects. The results showed that the LD50 of evodiamine and wyc-7-20 was, respectively, 27 μg/mL and 250 μg/mL on SH-SY5Y cells (Figure 1B). The cytotoxicity of wyc-7-20 was reduced by eightfold compared with that of evodiamine on SH-SY5Y cells. The LD50 of evodiamine and wyc-7-20 was, respectively, 5 μg/mL and 500 μg/mL on HepG2 cells (Figure 1C). The cytotoxicity of wyc-7-20 was reduced by a hundredfold compared with that of evodiamine on HepG2 cells. These results suggested that wyc-7-20 existed lower neurotoxicology and hepatotoxicity than evodiamine.

Wyc-7-20 Protects SH-SY5Y Cells Against H2O2 and AβOs

Both reactive oxidative stress (ROS) and AβOs are involved in AD pathological development. The protective effect of wyc-7-20 against H2O2 and AβOs was detected on SH-SY5Y cells. The results showed that wyc-7-20 revealed a protective effect against H2O2 at a dose of 0.00010 μg/mL (Figure 2A, n = 6, p < 0.01) and existed a similar protective effect from 0.00010 μg/mL to 10.0 μg/mL. However, no protective effect of evodiamine was detected. The dose of 0.10 μg/mL of wyc-7-20 was used to detect its effect against AβOs on SH-SY5Y, because it showed better repeatability than other doses (Figure 2A, n = 6, p < 0.0001).

The viability of SH-SY5Y cells treated with AβOs (15 μM) alone or AβOs and wyc-7-20 (0.10 μg/mL) were comparatively detected with a real-time cell analyzer. After a 69-h culture, CI of SH-SY5Y cells was obviously decreased by AβOs cytotoxicity compared with vehicle, while the wyc-7-20 significantly protected the decrease of CI caused by AβOs cytotoxicity (Figure 2B and C, n = 6, p < 0.05).

Wyc-7-20 Treatment Improves Cognitive Behavior of AD Mice

The dose of wyc-7-20 (0.1 μg/mL) was used for administration because it showed anti-H2O2 and anti-AβOs effects in in vitro bioactivity evaluation. Through calculation, the 3×Tg mice were treated with wyc-7-20 at 200 μg/kg via i.p every 2 days for a month. The mice of WT, vehicle control, and treatment group were, respectively, applied to Y maze test assay. The alternative percentage of the vehicle control mice was obviously decreased compared with the WT mice, while the wyc-7-20 treatment recovered the decrease of the alternative percentage of 3×Tg mice significantly (Figure 3A, n = 6, p < 0.05). The results suggested that wyc-7-20 treatment improved the working memory of the AD mice. After the Y maze assay, the mice were applied to the MWM assay. The result showed that wyc-7-20 treatment improved the learning ability indicated with increased platform latency compared with vehicle control (Figure 3B, n = 6, p < 0.05). The wyc-7-20 treatment increased the average value of number of crossing and quadrant occupancy, but without statistical significance (Figure 3C–E, n = 6).

Table 1 Primers Used for RT-qPCR

| Gene  | Forward Primers                     | Reverse Primers                      |
|-------|-------------------------------------|--------------------------------------|
| Gyp2e1| 5'-TGTCATCCTCCAAGGGTACAG-3'         | 5'-GCAGAAACAGTTCCATGCGG-3'           |
| Fabp3 | 5'-GGGAAACTCTACGTGACTTCA-3'         | 5'-ATTCACCTTGGAGACCCTTT-3'           |
| Fabp7 | 5'-GGCAGATGGTGCTGACTCT-3'           | 5'-TGCTAAGCTTGGAGACTCCA-3'           |
| S100b | 5'-GACCTCCAGCAGAAAGGTA-3'           | 5'-CTTCTCTGCTTGGTATTCCCT-3'          |
| Anparl| 5'-GGGTCCGCCCCGTGAACGATCA-3'        | 5'-TCAGAGCATCCTGTTCCTGCC-3'          |
| Pd95  | 5'-TGAGTTCAGCAGTGAACGAAA-3'         | 5'-GATTGCTGTCTGTGACCTCCGAG-3'        |
| Gapdh | 5'-GGTTCTATAAAATACGGACTG-3'         | 5'-CAATACGCCCCATCGTTCA-3'            |
Figure 1 Determinant the LD50 of wyc-7-20. Synthetic route for wyc-7-20 was as in (A). Reagents and conditions: (a): Na₂CO₃, hydroxylamine hydrochloride, H₂O, 70°C, 12 h, yield 90%. (b): chloroacetyl chloride, acetone, rt, 30 min, yield 89%. (c): toluene, reflux, 2 h, yield 83%. (d): 1-[4-(methylphenyl) carbonyl] piperazine, K₂CO₃, CH₃CN, reflux, 8 h, yield 88%. The SH-SYSY and HepG2 cells were seeded in 96-well plates respectively and cultured for 24 h. A series of concentrations (0.000010 μg/mL to 300.0 μg/mL) of wyc-7-20 and evodiamine were then added into 96-well plates respectively and continually cultured for 24 h. The median lethal dose (LD50) of wyc-7-20 on SH-SYSY cells (B) and on HepG2 cells (C) were detected by cell viability.

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Wyc-7-20 Treatment Alleviates Aβ and p-Tau Levels

The frozen brain sections from mice of WT, vehicle control, and treatment group were applied to immunofluorescence staining with Aβ antibody (6E10). The decrease of Aβ plaques and intracellular Aβ were observed, and Aβ covered area was significantly reduced by 70% in the hippocampus of wyc-7-20 treated mice compared with vehicle control (Figure 4A and B, n = 3, p < 0.05). Western blot showed that wyc-7-20 treatment reduced phosphorylation of Tau and Tau5 expression by 30% and 50%, respectively, compared with vehicle control (Figure 4C–E, n = 3, p < 0.05; Figures S2 and S3).

Wyc-7-20 Treatment Reverses Expression of AD-Related Genes

The total RNA was isolated from brain tissues of WT, vehicle control, and treatment group. A number of genes related to AD were selected to estimate the effect of wyc-7-20 on a molecular level, including the biological processes of metabolism (Cyp2e1 and Fabp3), astrocyte activation (S100b and Fabp7), neurotransmitter signaling (Ampar1) and synaptic plasticity (Psd95). The expression of the 6 genes were detected by RT-qPCR and normalized with Gapdh. The
expression of Cyp2e1, Fabp3, S100b and Fabp7 was significantly increased in the vehicle control mice (Figure 5A–D, n = 4, p < 0.05), suggesting that these genes could associate with pathological development in AD mice model. The wyc-7-20 treatment reversed the increased expression of Cyp2e1, Fabp3, S100b and Fabp7 (Figure 5A–D, n = 4, p < 0.05).

The alternative expression of Ampar1 and Psd95 was not detected in mice of WT, vehicle control, and treatment group (Figure 5E and F).

**Discussion**

Medications that have been on the market for AD are cholinesterase inhibitors (donepezil, rivastigmine, and galantamine) and NMDA receptor non-competitive inhibitors (memantine). Those medications were presented with cognitive improvement to some extent, as well as solved some issues such as limited blood-brain-barrier penetration and low selectivity. Toxicity and gastrointestinal adverse reaction problems continue to be a source of concern. The biggest problem comes with low effectiveness. Only a few patients respond to these medical treatments, and the clinical results are only statistically significant but without significant improvement in cognition and behavior assessment individually. Besides, none of them showed significant effectiveness in slowing the disease progression. Therefore, novel treatment is needed to develop.

In this study, we found a derivative of 1,2,4-Oxadiazole, wyc-7-20, which revealed low cytotoxicity and potent effect on the improvement of AD phenotypes in mice model. The cytotoxicity of wyc-7-20 on SH-SY5Y cells and HepG2 cells was reduced by eightfold and hundredfold compared to evodiamine (Figure 1), which showed wyc-7-20 has a lower cytotoxicity. wyc-7-20 showed a protective effect against the cytotoxicity of H2O2 and AβOs on SH-SY5Y (Figure 2A, p < 0.0001 and Figure 2C, p < 0.05). The Aβ plaques and phosphorylation of Tau were reduced significantly in the wyc-
Figure 4 Pathological observation of mice treated with wyc-7-20. The brain sections of mice from WT, 3×Tg and wyc-7-20 treatment group were stained with anti- Aβ (6E10, (A)). The Aβ covered area percentage in hippocampus were qualified by Image J software (B). The phosphorylation of TAU (AT-8 antibody) and TAU5 expression were detected by Western blot (C) and quantitatively analyzed by grey density with Image J software and normalized by GAPDH (D, E). n=3, *P<0.05 wyc-7-20 treatment vs 3×Tg.
7-20 treated AD mice (Figure 4, p < 0.05). The working memory and learning ability of AD mice were also improved by wyc-7-20 treatment (Figure 3, p < 0.05). The effective dosage of evodiamine was 100 mg/kg described in our previous work,\textsuperscript{25} while the effective dosage of wyc-7-20 was 200 μg/kg in this work. Compared with evodiamine, the effective dosage of wyc-7-20 was increased five hundredfold, suggesting that wyc-7-20 was a potential derivative on reduction of cytotoxicity and promoted to AD therapy.

Metabolic dysfunction has been the common pathological change in AD. The AD brain changed fuel from glucose to ketone bodies.\textsuperscript{31,32} The Cyp2e1 was regulated by the insulin signal pathway and involved in the ketone metabolism and ROS production.\textsuperscript{33,34} The increased expression of Cyp2e1 in AD mice could be one of the resources of ROS. The Fabp3 was a lipid metabolism-related biomarker of AD\textsuperscript{35} and was positively correlated with inflammation and activation of astrocytes of AD patients.\textsuperscript{36,37} The increased expression of Fabp3 in AD mice marked its dysfunction of lipid metabolism and immune state. The wyc-7-20 treatment reversed the increased expression of Cyp2e1 and Fabp3 (Figure 5A and B, p < 0.05), suggesting that wyc-7-20 could improve the lipid metabolism and reduction of ROS in AD mice. This result suggested that wyc-7-20 could decrease the amyloid plaques (Figure 4A and B, p < 0.05) and improve the activation of microglia. S100b is overexpressed in both AD patients and AD models, especially in the severely affected regions of the brain.\textsuperscript{38,39} Fabp7 is a newly found gene, which is involved in the proliferation of reactive astrocytes.\textsuperscript{40} Both S100b and Fabp7 are part of the proliferation and activation of astrocytes.\textsuperscript{40,41} The increased expression of S100b and Fabp7 in the brain of 3×Tg AD mice was significantly reversed by wyc-7-20 treatment (Figure 5C and D, p < 0.05), suggesting that wyc-7-20 could improve the activation of astrocytes.

**Conclusion**

Taking together, wyc-7-20 is a new compound with effects on improvement of AD phenotypes in mice model.
Ethics Approval

The study was conducted and approved by the Animal Care and Use Committee of the Institute of Laboratory Animal Science of Peking Union Medical College (ZLF180003) according to the China approved the animal protocol (ILAS-GC -2015-002) and National Institutes of Health Guide for the Care and Use of Laboratory Animals. Briefly, mice were maintained in isolated ventilated cages and feed with standard rodent diet of chow and water ad libitum, housed under a 12-hour light/dark cycle in a temperature (22–24°C) and humidity (40–60%) controlled room. All the mice were sacrificed through cervical vertebra removal for biochemical analysis. Efforts were made to minimize animal suffering and to reduce the number of animals used.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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