

Anti-epileptic mechanism of isopimaric acid from *Platycladi cacumen* based on network pharmacology, molecular docking and biological validation

YAN WANG^{1,2*}, YUN WANG^{1-3*}, CHANG LI¹⁻³, DONG LIU², YI CAI¹⁻³ and QIFU LI¹⁻³

¹Department of Neurology, The First Affiliated Hospital of Hainan Medical University, Haikou, Hainan 570102, P.R. China; ²Engineering Research Center of Tropical Medicine Innovation and Transformation of Ministry of Education, International Joint Research Center of Human-Machine Intelligent Collaborative for Tumor Precision Diagnosis and Treatment of Hainan Province, Hainan Key Laboratory for Research and Development of Tropical Herbs and Haikou Key Laboratory of Li Nationality Medicine, School of Pharmacy, Hainan Medical University, Haikou, Hainan 571199, P.R. China; ³Key Laboratory of Brain Science Research and Transformation in Tropical Environment of Hainan Province, Hainan Medical University, Haikou, Hainan 571199, P.R. China

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Abstract. *Platycladi cacumen* (PC) is derived from the dry twigs and leaves of *Platycladi orientalis* (L.) Franco and exerts anti-epileptic effects. However, its mechanism of action remains unknown. The present study explored the potential anti-epileptic components and mechanisms of PC. The primary active components and targets of PC were analyzed using network pharmacology and a lipopolysaccharide (LPS)-induced murine microglial cell line (BV2) was used to confirm anti-epileptic effects by detecting reactive oxygen species (ROS), apoptosis, inflammatory markers, cell migration and signaling pathways. A total of 13 core active components showed druggable properties, of which deoxypicrododophyllotoxin, hinokinin and isopimaric acid (IPA) were predicted to cross the blood-brain barrier. In total, 255 potential targets of these three compounds were predicted using SwissTargetPrediction and Similarity Ensemble Approach websites and 150 were associated with epilepsy. *In vitro* experiments confirmed that IPA significantly inhibited LPS-induced microglial oxidative stress and inflammation by decreasing the migration area, cellular ROS content, lactate dehydrogenase release and early phase of apoptosis. IPA also increased the mRNA expression of anti-oxidative enzymes (superoxide dismutase-1 and -2) and suppressed inflammatory

cytokines (interleukin-1 β and tumor necrosis factor- α). Furthermore, IPA phosphorylated AKT and mTOR proteins. Taken together, the present findings suggested that IPA is a potential anti-epileptic compound derived from PC.

Introduction

Epilepsy is a common neurological disorder caused by abnormal brain discharge, characterized by recurrent limb twitching and loss of consciousness. It affects ~1% of the global population. Currently, antiseizure medication (ASM) is the primary treatment for epilepsy (1). However ~30% of cases remain medically intractable, resulting in a heavy economic burden on patients and society (2). Therefore, it is necessary to develop novel ASMs that effectively control seizures.

Platycladi cacumen (PC), a widely used traditional Chinese medicine, is derived from dry twigs and leaves of *Platycladi orientalis* (L.) Franco. It is traditionally used to cool blood, stanch bleeding, dispel pathogenic winds, remove dampness, eliminate phlegm and raise hair and blacken hair (3). Recently, PC was shown to exert anti-inflammatory, antioxidative and neuroprotective effects (4). Aqueous extracts of PC suppress lipopolysaccharide (LPS)-induced intestinal inflammation by increasing colon length and inhibiting fecal occult blood, severe diarrhea and enteritis (5). PC carbonisate-derived nanoparticles inhibit ulcerative colitis induced by 2,4,6-trinitrobenzenesulfonic acid in rats by decreasing tumor necrosis factor- α (TNF- α) and interleukin-6 and upregulating interleukin-10 (4). Furthermore, denuded mice treated with water extract of PC for 4 weeks exhibit increased hair growth with increasing hair bulb size and dermis and epidermal thickness (6). A similar effect was found with volatile oil extracts of PC (7). Furthermore, PC exerts renoprotective effects by targeting renal organic anion transporters 1 and 3 to inhibit protein activity (8). A total of 43 compounds have been extracted and separated by 75% methanol from PC and grouped as organic acids, flavonoids, phenylpropanoids, volatile oils and tannins (9). Among them,

Correspondence to: Professor Yi Cai or Professor Qifu Li, Department of Neurology, The First Affiliated Hospital of Hainan Medical University, 31 Longhua Road, Haikou, Hainan 570102, P.R. China
E-mail: caiyi66817616@163.com
E-mail: lee-chief@163.com

*Contributed equally

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myricitrin, quercitrin and amentoflavone are the primary compounds (10) that ameliorate liver ischemia-reperfusion and (11) kidney injury (11) and inhibit platelet activation in arterial thrombosis (12) and human breast cancer (13).

The efficacy of PC against epilepsy has been reported in 'Effective Prescription for Epilepsy Treatment' (14). However, its anti-epileptic components and underlying mechanisms remain unclear. Therefore, in the present study, the anti-epileptic compound PC was explored using network pharmacology and *in vitro* experiments.

Materials and methods

Construction of an 'Herbs-Components-Targets' (H-C-T) network. The Traditional Chinese Medicine System Pharmacology Database (TCMSP) was used to identify the active ingredients of PC (15), of which, the components whose toxicokinetic absorption, distribution, metabolism and excretion (ADME) adhered to oral bioavailability (OB) $\geq 30\%$ and drug-likeness (DL) ≥ 0.18 were defined as the main compounds. Druggable compounds that may cross the blood-brain barrier (BBB) as predicted by SwissADME (swissadme.ch/index.php) were further used to identify targets on the SwissTargetPrediction (SWISS; new.swisstargetprediction.ch/) and similarity ensemble approach (SEA; sea.bkslab.org/) websites by using relative Canonical Simplified Molecular Input Line Entry System (SMILES) numbers.

Genes associated with epilepsy were obtained from GeneCards (version 4.9.0; genecards.org/). Overlapping genes between targets of the druggable compounds and epilepsy-associated targets were retrieved using VENN map (bioinformatics.psb.ugent.be/webtools/Venn/). The protein-protein interaction (PPI) network was analyzed using the protein-protein interaction networks functional enrichment analysis online tool (STRING; string-db.org/) and core genes were obtained using the CytoNCA of Cytoscape3.9.1 (cytoscape.org/) with the criteria of two-fold the median value of degree centrality (DC), median values of betweenness centrality (BC) and closeness centrality (16). The H-C-T network of PC was constructed using Cytoscape3.9.1.

Gene functions and pathway analysis. Gene Ontology (GO) biological process, cellular component and molecular function and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were analyzed among the overlapping genes using the web-based tool DAVID v6.8 (david.ncifcrf.gov/tools.jsp) (17). $P < 0.05$ (Bonferroni-corrected) was considered to indicate statistical significance.

Cell culture and proliferation assay. The murine microglial cell line BV2 was purchased from Procell Life Science & Technology Co., Ltd. and cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (Clark Bioscience) and 1% streptomycin/penicillin (Biosharp Life Sciences) in a humidified incubator with 5% CO₂ at 37°C.

Cell proliferation was assessed using Cell Counting Kit-8 (CCK8; Dojindo Molecular Technologies, Inc.). Briefly, cells (1×10^4 /ml) were seeded and cultured in 96-well microplates for 24 h. Cells were treated with isopimaric

acid (IPA, Sigma-Aldrich; Merck KGaA; 0.1, 1.0, 10.0, 100.0 and 1,000.0 μ M) for 24 h, followed by incubation with 10 μ l CCK8 reagent for 1 h all at 37°C. Absorbance was measured by a microplate reader (BioTek Instruments, Inc.; EPOCH2NS) at 450 nm. Survival rate was calculated as follows (18): Survival rate % = absorbance of IPA/absorbance of control $\times 100\%$.

Wound healing assay. Confluent BV2 cells (90%) were scratched using a pipette tip and washed three times with PBS to remove non-adherent cells. The cells were incubated with LPS (1 μ g/ml; Sigma-Aldrich; Merck KGaA) in the presence or absence of 0.0, 0.1, 1.0, 10.0 or 100.0 μ M IPA for 24 h at 37°C. Images of the central cell-free zone before and after treatment were obtained by light field microscopy (magnification, $\times 200$; Zeiss X-Cite; Carl Zeiss AG) (19). The migratory area was calculated as follows: Migratory area (%) = [(area at 0 h - area at 24 h)/area at 0 h] $\times 100\%$.

LDH assay. The medium of cells treated with LPS in the presence or absence of IPA was collected to determine the released LDH content using assay kits (no. A020-2-2; Nanjing Jiancheng Bioengineering Institute) as previously described (20).

Flow cytometric analysis of cellular reactive oxygen species (ROS). Following treatment with LPS in the presence or absence of IPA for 24 h, cells were incubated at 37°C with 10 μ M 2',7'-dichlorofluorescein diacetate (MedChemExpress) diluted in DMEM for 30 min in the dark. Images were captured using a fluorescence microscope (Zeiss X-Cite; Zeiss AG) and mean intensity was measured using a flow cytometer (NovoCyte; Agilent Technologies, Inc.) in the fluorescein isothiocyanate (FITC) channel.

Annexin V-FITC/propidium iodide (PI) analysis for apoptosis. BV2 cells were digested 37°C for 5 min using trypsin, followed by incubation with Annexin-FITC and PI for 5 min at room temperature (Boster Biological Technology). Fluorescence intensities were detected using a flow cytometer (NovoCyte) with FITC and PI channels, as previously described (21). A total of four populations of cells were distinguished: Viable (no staining), early apoptosis (Annexin V⁺PI⁻), late apoptotic cells (Annexin V⁺PI⁺), and necrotic (Annexin V⁻PI⁺) cells. Apoptosis was determined as early + late apoptosis.

Determination of mitochondrial membrane potential (MMP). Cells were treated with IPA and LPS for 24 h, followed by incubation with 500 μ l JC-1 working solution in the dark (Beyotime Institute of Biotechnology) for 20 min at 37°C. Images were obtained using a fluorescence microscope in the FITC and PI channels (magnification, $\times 200$; Zeiss X-Cite; Carl Zeiss AG).

Reverse transcription-quantitative (RT-q)PCR. Total RNA from BV2 cells was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse-transcribed to cDNA using MonScript Reverse Transcriptase (cat. no. MR05101; Monad Biotech Co., Ltd.), followed by SYBR Green PCR (cat. no. MQ00401; Monad Biotech Co., Ltd.), according to the manufacturer's protocol as previously described (21). Relative

Table I. Sequence and length of primers.

Gene	Forward, 5'→3'	Reverse, 5'→3'	Length, bp
Actin	CCACAGCTGAGAGGGAAATC	AAGGAAGGCTGGAAAAGAGC	193
SOD-1	CCATCAGTATGGGGACAATACA	GGTCTCCAACATGCCTCTCT	109
SOD-2	GACCCATTGCAAGGAACAA	GTAAGTAAAGCGTGCTCCACAC	69
IL-1β	TGCCACCTTTTGACAGTGATG	GGAGCCTGTAGTGCAGTTGT	351
TNF-α	GTAGCCACGTCGTAGCAA	GTGAGGAGCACGTAGTCGG	191
Arg-1	GAACACGGCAGTGGCTTTAAC	TGCTTAGCTCTGTCTGCTTTGC	155

SOD, superoxide dismutase; Arg, arginase.

Table II. Antibody information.

Antibody	Supplier	Cat. no.	Dilution
Rabbit anti-TNF-1α	Cell Signaling Technology, Inc.	8184	1:1,000
Rabbit anti-IL-1β	Cell Signaling Technology, Inc.	12703S	1:1,000
Mouse anti-AKT	Proteintech Group, Inc.	60203-2	1:5,000
Rabbit anti-p-AKT	Proteintech Group, Inc.	80455-1-RR	1:5,000
Rabbit anti-mTOR	Abcam	ab2732	1:5,000
Mouse anti-p-mTOR	Proteintech Group, Inc.	67778-1	1:2,000
Mouse anti-PI3Kα	Proteintech Group, Inc.	67071-1-lg	1:1,000
Mouse anti-PI3Kβ	Proteintech Group, Inc.	67644-1-lg	1:5,000
Rabbit anti-GAPDH	Proteintech Group, Inc.	10494-1-AP	1:6,000

PI3K, phosphatidylinositol 3-kinase; p-, phosphorylation.

expression levels of target genes were calculated based on the $2^{-\Delta\Delta C_q}$ method using actin as a reference housekeeping gene (22). The primer sequences are listed in Table I.

Western blotting. Total protein was extracted from BV2 cells using a cell lysis buffer (cat. no. P0013, Beyotime Institute of Biotechnology) with a phosphatase inhibitor, while concentrations of proteins were determined by bicinchoninic acid method (Wuhan Boster Biological Technology, Ltd.). Protein lysates (50 μg) were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinyl difluoride membrane (MilliporeSigma) via electroblotting. Each blot was blocked by QuickBlock (cat. no. P0256; Beyotime Institute of Biotechnology) for 15 min at room temperature and incubated with primary antibodies overnight at 4°C (Table II). Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Proteintech Group, Inc.; cat. no. 20000858; 1: 2,000; cat. no. 20000757; 1:5,000) for 1 h at 37°C. The blots were visualized by the ChemiDoc XRS imaging system (Bio-Rad Laboratories, Inc.) with BeyoECL kit (cat. no. 081723240119; Beyotime Institute of Biotechnology) and quantified using ImageJ Software (v1.52a; National Institutes of Health).

Molecular docking. The crystallographic structure of AKT was obtained from the Protein Data Bank (PDB code: 4GV1) (23) and docking by using Schrödinger (version 2015) (24). Briefly,

the Protein Preparation Wizard and Receptor Grid Generation modules were used to prepare the proteins. Ionization-generated possible states of the LigPrep module were set at a target pH of 7.0 ± 2.0 to prepare IPA to dock flexibly into the ligand site using a Ligand Docking module in standard precision mode, as previously described (25).

Statistical analysis. Data are presented as the mean \pm SD. Normally distributed data were analyzed using the Shapiro-Wilk test and one-way ANOVA followed by Dunn's post hoc test for multiple groups using GraphPad Prism (version 9.0.0; Dotmatics) (26). Non-normally distributed data were analyzed using Kruskal-Wallis test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Targets prediction of PC and visualization of H-C-T network. A total of seven primary compounds were obtained from the TCMSP, of which hinokinin, IPA and deoxypicropodophyllotoxin (DPT) exhibited the potential to cross the BBB (Table III; Fig. 1A). Based on SMILES numbers of these three components, 255 potential targets of PC were identified by target fishing from the SWISS and SEA databases, of which 150 were associated with epilepsy (Fig. 1B). These composite targets were input into STRING to construct a PPI network with connected targets (combined score > 0.7), including 259 nodes and 308 edges

Table III. Characteristics of seven active constituents derived from PC.

ID no.	Molecule	MW (g/mol)	DL	OB, %	BBB-permeable
MOL000098	Quercetin	302.25	0.28	46.43	No
MOL000358	β -sitosterol	414.79	0.75	36.91	No
MOL000422	Kaempferol	286.25	0.24	41.88	No
MOL002005	Hinokinin	354.38	0.64	56.5	Yes
MOL002032	DNOP	390.62	0.40	40.59	No
MOL002034	(5aR,8aS,9R)-9-(3,4,5-trimethoxyphenyl)-5a,6,8a,9-tetrahydro-5H-isobenzofurano[5,6-f][1,3]benzodioxol-8-one (Deoxypicropodophyllotoxin)	398.44	0.83	52.70	Yes
MOL002039	Isopimaric acid	302.45	0.28	36.20	Yes

MW, molecular weight; DL, drug-like; OB, oral bioavailability; BBB, blood-brain barrier; DNOP, dioctyl phthalate.

(Fig. 1C). A total of 13 targets, including TNF, tumor protein (TP53), estrogen receptor 1 (ESR1), prostaglandin-endoperoxide synthase 2 (PTGS2), microtubule affinity regulating kinase 3 (MARK3), peroxisome proliferative activated receptor gamma (PPARG), caspase 3 (CASP3), B-cell lymphoma-2 (BCL2), glycogen synthase kinase 3 beta (GSK3B), mammalian target of rapamycin (mTOR), protein tyrosine phosphatase non-receptor type11 (PTPN11), sirtuin1 (SIRT1) and murine double minute 2 (MDM2) exceeded the values (two-fold of DC, median of BC and closeness centrality; Fig. 1D). Among these, BCL2, GSK3B, and mTOR are potential anti-epileptic targets for hinokinin; CASP3, mTOR, and SIRT1 for DPT; and TNF, TP53, ESR1, PTGS2, MAPK3, PPARG, PTPN11, and MDM2 for IPA. In particular, TNF showed the highest subgraph centrality value.

GO and KEGG enrichment analysis. GO and pathway enrichment analyses were performed for overlapping targets (150 genes) that were significantly enriched 'protein phosphorylation', 'negative regulation of apoptotic process', 'response to xenobiotic stimulus', 'protein autophosphorylation' and 'peptidyl-serine phosphorylation' in the biological processes. In terms of cellular component, the overlapping targets were enriched in the 'membrane raft', 'macromolecular complex', 'dendrite', 'cell surface', and 'receptor complex'. With respect to molecular function, the core targets were enriched in 'protein serine/threonine/tyrosine kinase activity', 'protein serine/threonine kinase activity', 'kinase activity', 'protein tyrosine kinase activity', and 'RNA polymerase II transcription factor activity, ligand-activated sequence-specific DNA binding' (Fig. 1E). A total of 150 genes were enriched in 140 pathways, of which 'pathways in cancer', 'PI3K-Akt signaling pathway', 'pathways of neurodegeneration-multiple diseases', 'cAMP signaling pathway' and 'MAPK signaling pathway' were the most enriched (Fig. 1F).

IPA inhibits elevation of ROS production and migration of murine microglia cells induced by glutamate and LPS. IPA serves a role in pathological processes, including anti-bacterial activity (27) and anti-NLR family, pyrin domain containing protein 3 (NLRP3) inflammasome (28) and anti-Alzheimer's disease effects (29,30). IPA activates large-conductance Ca^{2+} -activated K^{+} channels (31,32) by

targeting gamma-aminobutyric acid (GABA) receptors to induce chloride ion currents (33). Therefore, it was hypothesized that IPA may be a target compound to treat epilepsy. Hence, glutamate- and LPS-induced excitotoxicity and neuroinflammation in murine microglia cells BV2 were examined to determine the anti-epileptic effects of IPA, as previously described (34).

IPA (0.1-1,000.0 μM) was used to verify its effect on the survival of BV2 cells. Concentrations of IPA from 0.1 to 100.0 μM did not affect the survival rate of BV2 cells and were applied in subsequent experiments (Fig. 2A and B). BV2 cells treated with glutamate (5 mM) for 12 h and LPS (1 $\mu\text{g}/\text{ml}$) for 24 h notably suppressed the survival rate. However, co-administration of IPA did not improve the survival of BV2 cells (Fig. 2C and D) but significantly suppressed the production of ROS induced by glutamate (Fig. 2E and F) and LPS (Fig. 2G and H).

IPA significantly inhibited the wound closure of BV2 induced by LPS (0.1, 1.0, 10.0 and 100 μM IPA corresponded to 88.10 ± 20.17 , 91.55 ± 28.29 , 83.03 ± 11.79 and $70.55 \pm 29.71\%$, respectively, compared with $146.60 \pm 7.19\%$ migration area in the LPS group; Fig. 2I and J) and decreased LDH release (0.1, 1.0, 10.0 and 100.0 μM IPA doses corresponded to 352.93 ± 72.37 , 389.63 ± 75.72 , 319.76 ± 40.31 , and 244.11 ± 31.44 , respectively, compared with 495.12 ± 47.63 U/l in the LPS group; Fig. 2K).

IPA suppresses LPS-induced apoptosis in BV2 cells. Treatment with IPA in the range of 0.1-100.0 μM prevented the LPS-induced late phase of apoptosis (LPS, $9.94 \pm 6.05\%$; 0.1 μM , $3.47 \pm 1.30\%$; 1 μM , $4.19 \pm 1.58\%$; 10 μM , $4.40 \pm 2.10\%$; 100 μM , $4.33 \pm 2.88\%$; Fig. 3A-C). It was confirmed by MMP that BV2 cells treated with IPA showed decreased JC-1 monomers compared with the LPS group (Fig. 3D).

IPA suppresses mRNA expression of anti-oxidative genes including superoxide dismutase (SOD)-1 and SOD-2, inflammatory genes (IL-1 β and TNF- α) and M2-polarization genes (Arg-1) in LPS-treated BV2 cells. To explore the mechanism of action of IPA, mRNA expression of SOD-1 and SOD-2, which indicate ROS overload (35), was assessed. IPA at concentrations of 1, 10, and 100 μM significantly increased mRNA expression of SOD-1 and



Figure 1. Network pharmacological study on the anti-epileptic effect of *Platycladi cacumen*. (A) Analysis of druggable compounds. A total of 86 compounds were obtained from TCMSP database, of which seven fulfilled the criteria $OB \geq 30\%$ and $DL \geq 0.18$. Hinokinin, DPT and IPA were predicted to cross BBB. (B) Predicted targets of hinokinin, DPT and IPA from SwissTarget and SEA database. (C) Venn diagram of overlapping target genes of compounds and epilepsy-associated genes. (D) Hub gene analysis by CytoNCA of 150 overlapping genes. (E) GO functional enrichment from 150 overlapping genes. (F) Pathways analysis of 50 overlapping genes. TCMSP, Traditional Chinese Medicine System Pharmacology Database; OB, oral bioavailability; DL, drug-likeness; DL, deoxypicropodophyllotoxin; IPA, isopimaric acid; BBB, blood-brain barrier; SEA, similarity ensemble approach; BP, biological processes; CC, cellular component; MF, molecular function; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology.

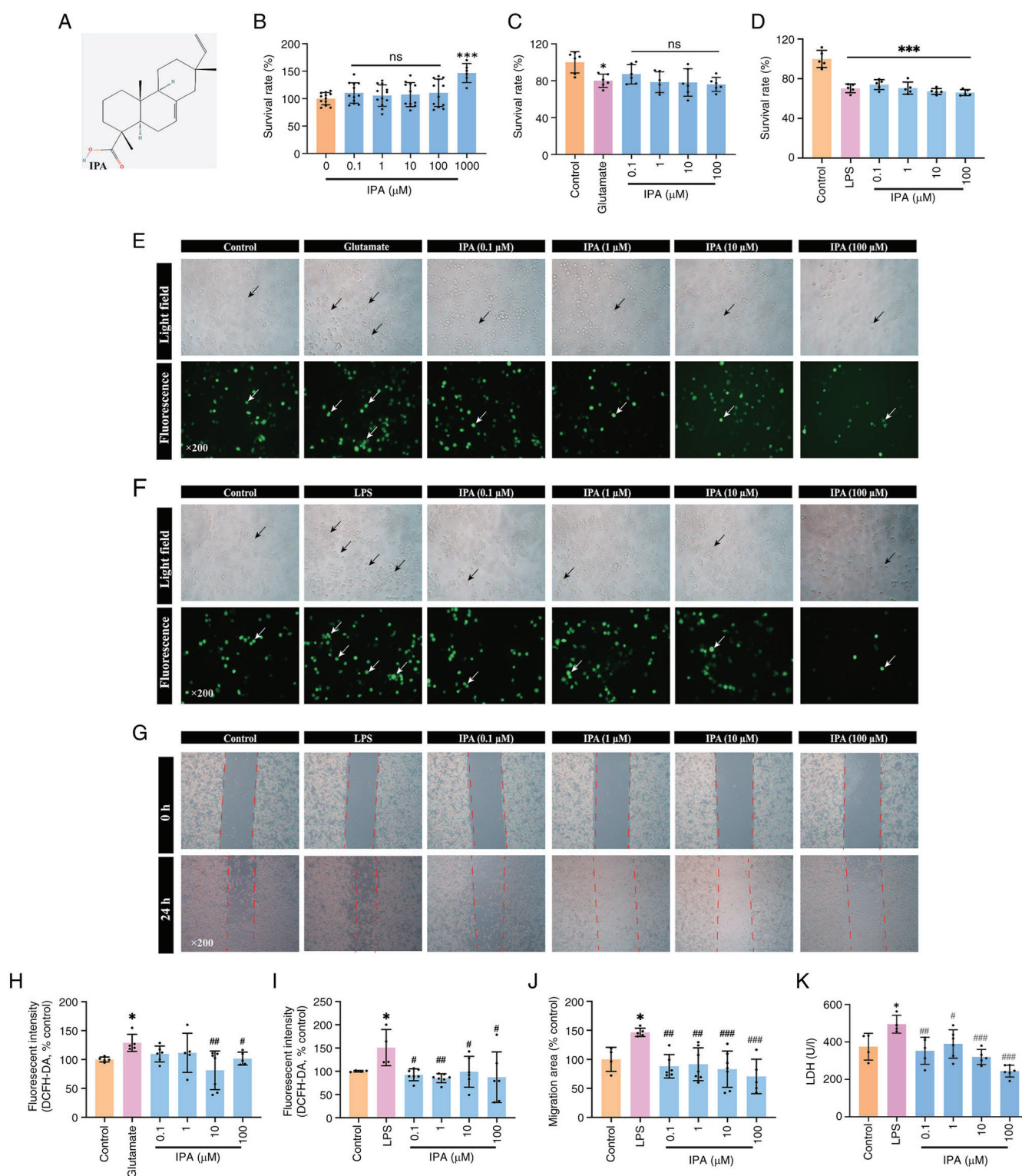


Figure 2. Anti-epileptic effects of IPA in LPS-induced BV2 cells. (A) Compound structure of IPA. (B) Survival rate of BV2 cells treated in the presence or absence of IPA for 24 h, compared with the 0 μ M group. (C) Survival rate of BV2 cells treated with glutamate without or with IPA for 12 h. (D) Survival rate of BV2 cells treated with LPS without or with IPA for 24 h. (E) DCFH staining of the BV2 cells induced by glutamate without or with IPA for 12 h. (F) DCFH staining of the BV2 cells induced by LPS without or with IPA for 24 h. (G) Migration of BV2 cells induced by LPS without or with IPA for 24 h. (H) Analysis of ROS content in BV2 cells induced by glutamate without or with IPA. (I) ROS content in BV2 cells induced by LPS in the presence or absence of IPA. (J) Migration area and (K) LDH release of BV2 cells induced by LPS in the presence or absence of IPA. $n=4-7$. * $P<0.05$, *** $P<0.001$ vs. control; # $P<0.05$, ## $P<0.01$, ### $P<0.001$ vs. LPS or glutamate group. IPA, isopimaric acid; LPS, lipopolysaccharide; ROS, reactive oxygen species; LDH, Lactic dehydrogenase; ns, no significance.

SOD-2 (Fig. 4A and B). LPS induced significant increases of the mRNA expression of inflammatory genes including *IL-1 β* (Fig. 4C) and *TNF- α* (Fig. 4D), while 100 μ M of

IPA significantly decreased the mRNA expression of *IL-1 β* and *TNF- α* . Furthermore, it was demonstrated that LPS suppressed the mRNA expression of *Arg-1*, a specific

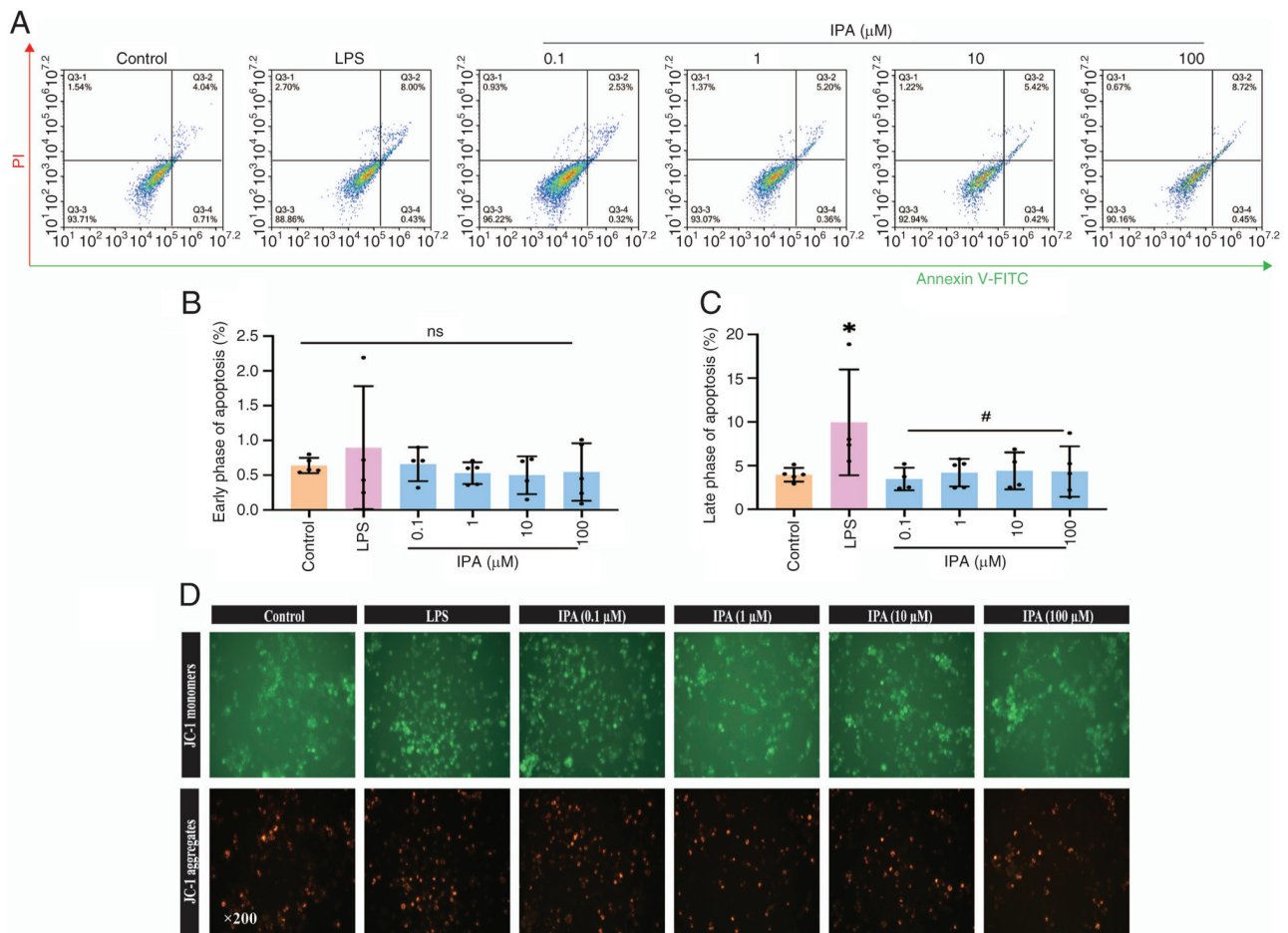


Figure 3. IPA inhibits LPS-induced late apoptosis of BV2 cells. (A) Representative flow cytometry. (B) Early and (C) late apoptotic rate. (D) Representative JC-1 staining. n=4 or 5. *P<0.05 vs. control; #P<0.05 vs. LPS. IPA, isopimaric acid; LPS, lipopolysaccharide; ns, no significance.

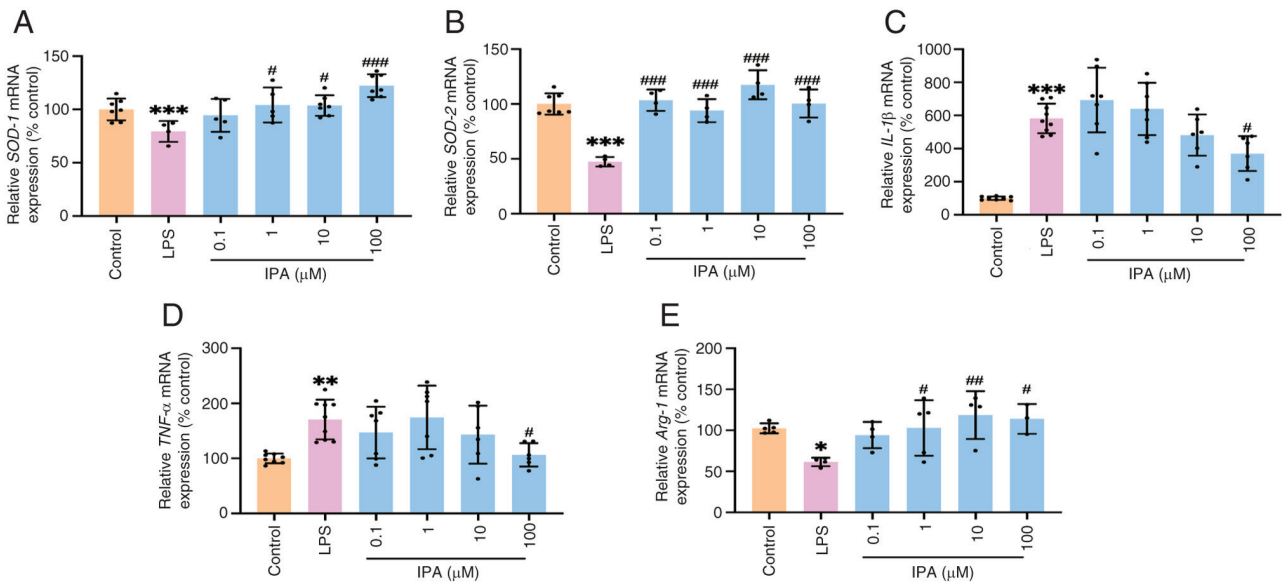


Figure 4. mRNA expression of genes associated with anti-oxidation, inflammation and polarization. mRNA expression of (A) SOD-1 (B) SOD-2 (C) IL-1β, (D) TNF-α and (E) Arg-1. n=3-9, *P<0.05, **P<0.01, ***P<0.001 vs. control; #P<0.05, ##P<0.01, ###P<0.001 vs. LPS. SOD, superoxide dismutase; Arg, arginase; IPA, isopimaric acid; LPS, lipopolysaccharide.

surface phenotype marker of M2 (36). IPA at concentrations of 1, 10, and 100 μ M significantly increased the

mRNA expression of *Arg-1*, indicating that IPA induced the polarization of BV2 to M2 (Fig. 4E).

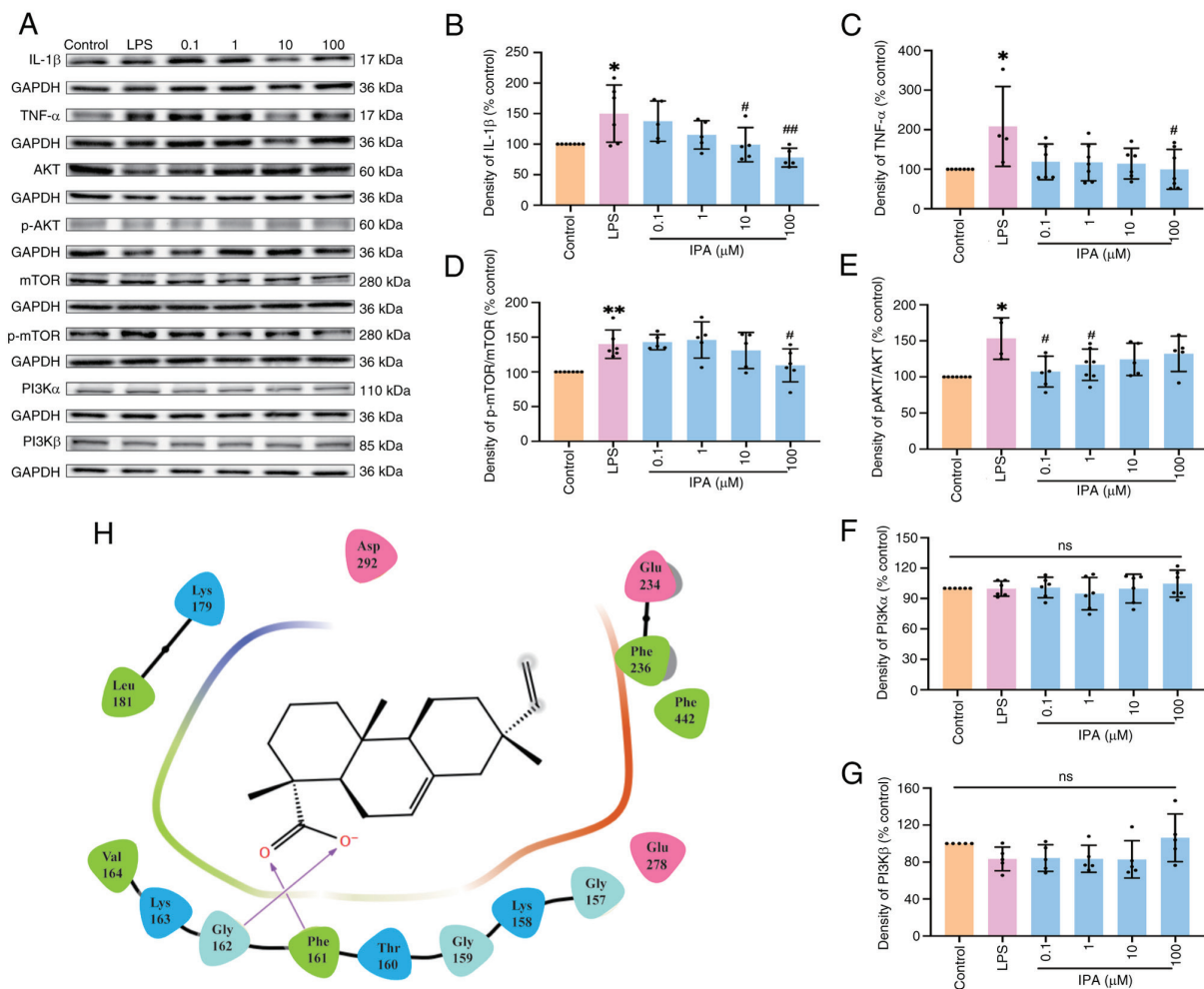


Figure 5. Protein expression of potential targets. (A) Representative blots for IL-1 β , TNF- α , AKT, p-AKT, mTOR, p-mTOR, PI3K α and PI3K β . Protein expression analysis of (B) IL-1 β (C) TNF- α , (D) ratio of mTOR and p-mTOR, (E) ratio of AKT and p-AKT, (F) PI3K α and (G) PI3K β . (H) Binding model of IPA with active pocket of AKT. $n=4-7$, * $P<0.05$, ** $P<0.01$ vs. control; # $P<0.05$, ## $P<0.01$ vs. LPS. IPA, isopimaric acid; LPS, lipopolysaccharide; PI3K, phosphatidylinositol 3-kinase; p-, phosphorylation.

IPA suppresses protein expression of IL-1 β and TNF- α by inhibiting the phosphorylation of hyperactive mTOR and AKT. Protein expression of hub (TNF- α and mTOR) and PI3K/AKT signaling pathway genes (AKT and p-AKT; Fig. 5A) were assessed. IPA inhibited protein expression of IL-1 β (Fig. 5B) and TNF- α (Fig. 5C) compared with the LPS group. IPA also suppressed phosphorylation of mTOR (Fig. 5D) and AKT (Fig. 5E) but not of phosphatidylinositol 3-kinase (PI3K) α and β (Fig. 5F and G). Furthermore, molecular docking results confirmed that two oxygen atoms of carboxyl in IPA docked on the key Phe161 and Gly162 residue of AKT, which may interrupt the phosphorylation of AKT (37) (Fig. 5H).

Discussion

A total of ~40 antiepileptic drugs have been used to treat epileptic patients in clinical settings (38). However, most control the occurrence of acute seizures and do not exert a true antiepileptogenic effect. Currently, ~30% of patients with epilepsy experience uncontrollable seizures (39). Therefore, exploring novel antiepileptic drugs that impede epileptogenesis is vital for treating refractory epilepsy. Chinese herbal medicines including *Gastrodia elata*, *Uncaria rhynchophylla*,

Acrori tatarinowii, *Paeonia lactiflora*, *Bupleurum Chinese* and PC have been used to treat seizures and epilepsy for thousands of years (40-42). The present study demonstrated that three compounds from PC, hinokinin, DPT and IPA showed druggability in crossing the BBB. In total, 150 predicted targets were associated with epilepsy, suggesting that hinokinin, DPT and IPA are potential components of PC against epilepsy.

Microglia are the primary glial cells in the central nervous system and act as immune protectors to maintain stability of the nerve cell microenvironment. Upon abnormal stimulation, microglia are transformed, exhibiting cellular structures varying from ramified to amoeboid, and enhancing migration to the injured region (43), where they induce release of inflammatory cytokines (IL-1 β and TNF- α), and cause inflammation (44). Microglial activation and inflammation have been observed in the brain of patients with refractory epilepsy (45,46), which increases neuronal excitability and contributes to epileptogenesis (47). In the present study, IPA, a diterpenoid compound separated from PC (48), alleviated glutamate- and LPS-induced oxidative stress and inflammation in BV2 cells, confirming previous studies where IPA not only inhibited production of inflammation protein NF- κ B in HBEC3-KT (*Homo sapiens* lung and bronchial epithelial

cells), MRC-5 (*Homo sapiens* lung fibroblasts), and THP-1 cells (*Homo sapiens* peripheral blood monocyte) (49), but also suppressed the proliferation and metastasis of breast cancer cells including (MDA-MB-231 and MCF-7) via mitochondrial oxidative phosphorylation signaling pathways (50). In particular, IPA significantly increased mRNA expression of anti-oxidative kinases (SOD-1 and SOD-2) and decreased gene expression of inflammatory factors (IL-1 β and TNF- α), suggesting an anti-inflammation role in microglia.

An increasing number of studies have confirmed that hyperactive mTOR is involved in inflammation and apoptosis of microglia during epileptogenesis and is a potential target for epileptic treatment (51,52). Somatic mTOR variants, including p.C1483Y and p.C1483R, have been identified in patients with refractory epilepsy and focal cortical malformation (53), while, kainic acid- and LPS-induced seizures significantly activate mTOR in rats (54,55). The activation of mTOR in microglia enhances inflammatory responses (56). Furthermore, the PI3K/Akt pathway is key for mTOR-involved cell survival and migration (57,58). The present study demonstrated that IPA significantly inhibited phosphorylation of mTOR, confirming a previous study showing that inhibiting the PI3K/Akt/mTOR pathway prevents microglial apoptosis (59). As previous studies have demonstrated that protein expression of PI3K α and β is significantly increased in acute and chronic epilepsy, independent of phosphorylation levels (60,61), the present study only analyzed the protein expression of PI3K α and β . However, the present study did not find any changes to PI3K α and β . Further analysis by molecular docking suggested that IPA may directly combine with AKT at the Phe161 and Gly162 residues and suppress activation of AKT, in line with a previous study that demonstrated residue Phe161 serves a vital role on AKT (37). Overall, the present study suggested that IPA inhibited LPS-induced neuroinflammation via the Akt/mTOR pathway. However, lack of data on the selectivity and specificity of IPA for AKT and mTOR in anti-epileptic activity is a limitation of the present study. Hence, their direct association should be investigated in the future.

In summary, IPA may be a potential anti-epileptic compound in PC that acts by suppressing neuroinflammation, apoptosis and polarization via the Akt/mTOR pathway. These findings indicate that IPA may be a novel anti-epileptic drug.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YaW conceived and designed the study, analyzed data, wrote the manuscript and constructed figures. YuW, CL and DL designed the experiments. YuW performed experiments and constructed figures. YaW, YC, and QL confirm the authenticity of all the raw data. YC and QL designed the experiment, wrote the manuscript, and supervised the study. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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