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ORIGINAL ARTICLE

Synthesis of Baicalin Carboxylate Derivatives and their Structure–Activity Relationship Analysis of their Inhibitory Activity on BVDV NS5B Polymerase

Ying Zong[1], Yu H. Sun[1], Yi Che[2], Hui Wang[1], Kun Shi[1], Zhong M. He[1], Rui Du[1,3]

[1]College of Chinese Medicinal Material, Jilin Agricultural University, Changchun, Jilin Province, [2]The First Affiliated Hospital to Changchun University of Chinese Medicine, Changchun, Jilin Province, [3]Jilin Province Meihua Deer Production and Product Application Research Laboratory, Changchun, Jilin Province, China

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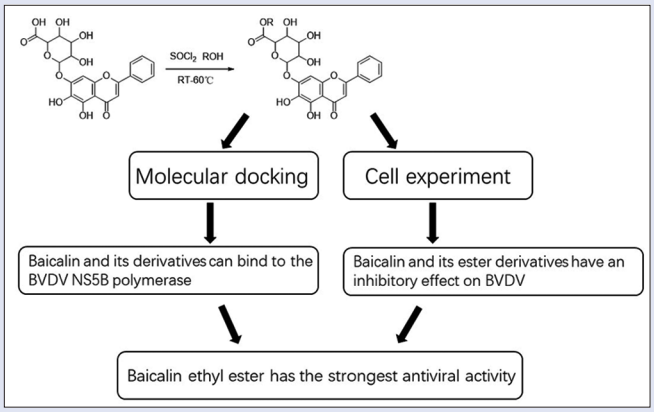
ABSTRACT

Background: Bovine viral diarrhea virus (BVDV) has a serious impact on the global livestock industry; however, there are no specific therapeutic drugs for BVDV, so the development of anti-BVDV drugs is a research priority. **Objectives:** To investigate whether baicalin and its ester derivatives are active against BVDV non-structural protein 5B (NS5B). **Materials and Methods:** We modified the sugar chain part of the structure of baicalin by esterifying the carboxyl group at the 6-position of 7-β-d-glucuronide and by introducing alkyl groups of different lengths. The binding and *in vitro* activity of the baicalin ester derivatives with BVDV NS5B polymerase was determined using molecular docking, molecular dynamics, Cell Counting Kit-8 (CCK-8) assays, and real-time RT-PCR. **Results:** The following six baicalin ester derivatives were obtained: baicalin methyl ester, baicalin ethyl ester, baicalin propyl ester, baicalin butyl ester, baicalin hexyl ester, and baicalin heptyl ester. Molecular docking, molecular dynamics, CCK-8 assays, and real-time RT-PCR showed that baicalin and its derivatives could bind to BVDV NS5B polymerase, with baicalin ethyl ester showing the best binding ability and antiviral activity. **Conclusion:** Baicalin and its ester derivatives exert an inhibitory effect on BVDV by targeting the BVDV NS5B polymerase, and this effect shows a structure–activity relationship. The results provide an important theoretical basis for the further development of anti-BVDV drugs.

Key words: Baicalin, bovine viral diarrhea virus, esterification reaction, inhibitory activity, molecular docking, NS5B polymerase

SUMMARY

- Baicalin and its ester derivatives exert anti-BVDV replication effects by targeting BVDV NS5B polymerase.
- The structure of baicalin derivatives has a certain relationship with their antiviral activity.



Abbreviations used: CCK-8: Cell Counting Kit-8; PI: persistent infection; RdRp: RNA-dependent RNA polymerase; HIV: human immunodeficiencyvirus; DMSO: dimethyl sulfoxide; MDBK: Madin-Darby bovine kidney; NDV: Newcastle disease virus; IC_{50}: half-maximal inhibitory concentration of a substance; PBS: phosphate-buffered saline; HBV: hepatitis B virus; TNTase: terminal nucleotidyl transferase; RSV: respiratory syncytial virus; $TCID_{50}$: median tissue culture infective dose; DENV: dengue fever virus; Gly: glycine; Asp: aspartate; Gln: glutamine; Trp: tryptophan; Glu: glutamic acid; Asn: asparagine; Cys: cysteine

Correspondence:
Dr. Rui Du,
Jilin Agricultural University, Changchun, Jilin Province 130118, China.
E-mail: durui197101@sina.com

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INTRODUCTION

Bovine Viral Diarrhea Virus (BVDV) is an RNA virus whose RNA is encapsulated in a capsid and phospholipid bilayer, which can cause mucosal disease, congenital malformations, and other manifestations.[1] BVDV infection in calves can cause persistent infection (PI), and cattle with PI can transmit BVDV to susceptible animals such as deer, sheep, and pigs.[2] BVDV outbreaks cause serious economic losses to the cattle industry.[3] Although some developed countries have achieved good results using BVDV vaccination,[4] there are still many countries where adverse reactions have been found after application of the vaccine.[5] Therefore, the development of drugs to treat BVDV infection has become a research hotspot. BVDV non-structural protein 5B (NS5B) has RNA-dependent RNA polymerase (RdRp) and terminal transferase (TNTase) activities and is mainly implicated in genomic transcription and duplication of the virus. NS5B is conserved in veterinary viruses because it is at the 3' end of the BVDV genome.[6] Therefore, research into anti-BVDV drugs targeting NS5B has a broad developmental prospect.

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Baicalin, the main element of *Scutellaria*, a commonly used herbal remedy to relieve fever, is an important flavonoid with significant pharmacological effects, including immunomodulation, anti-inflammatory, antibacterial, antiviral, antioxidant, and anti-neoplastic activities.[7-17] In particular, the antiviral effect of baicalin has attracted the attention of scholars at home and abroad in recent years. Baicalin has a broad-spectrum antiviral effect and has inhibitory effects on influenza virus, Zika virus, respiratory syncytial virus (RSV), Newcastle disease virus (NDV), dengue virus (DENV), human immunodeficiency virus (HIV), and hepatitis B virus (HBV).[18-24] Typically, antiviral compounds work effectively through solubilization in the extracellular medium, infiltration into the cell membrane, aggregation, and interaction with the target in four steps, with the lipophilic fraction playing a crucial role.[25] However, baicalin is poorly absorbed when administered orally because the three benzene rings in the structure form a stable conjugated system with high polarity and low permeability; therefore, the blood concentration is extremely low and its absolute bioavailability is only 2.2% ± 0.2%.[26,27]

The current derivatives of baicalin mainly include esterified derivatives,[28-30] hydroxyethyl (propyl)-based derivatives,[28] glycosylated derivatives,[31] and methylated derivatives.[32] A study reported that the anti-HIV activity was lost when the 6-position hydroxyl group in the structure of baicalin sapogenins was modified,[31] and also when the 6position hydroxyl group was substituted first in the hydroxyethylation reaction,[28] indicating its high reactivity. Therefore, the 6-position hydroxyl group is an important reactive group and this site should not be modified. In the present study, the hydroxyl group at positions 5 and 6 was not modified and the *o*-diphenol hydroxyl group was retained. Instead, the 6position carboxyl group of glucuronic acid was modified by an esterification reaction, and alkyl groups of different lengths were introduced to form a range of baicalin ester derivatives, with the aim of reducing the polarity of baicalin, improving its ability to penetrate phospholipid membranes,[33,34] increasing its oral bioavailability, and improving its penetration into virusinfected host cells to exert its antiviral effect.

Molecular docking is one of the methods used during drug design, which can predict and assess the reaction between the activity of ligands and receptors and the strength and mode of their binding with the help of computerized molecular simulation techniques. In this study, we found that baicalin ester derivatives would bind well to BVDV NS5B polymerase according to the computer simulation technique. In addition, the binding power of baicalin ester derivatives to BVDV NS5B polymerase varied depending on the alkyl group introduced in the sugar chain part of baicalin. Therefore, the structure relationship between the obtained baicalin ester derivatives and NS5B polymerase inhibitory activity was also investigated, and the activity was verified. This study provides a scientific reference for further development and utilization of baicalin and the development of new drugs against BVDV infection.

MATERIALS AND METHODS

Reagents

The following reagents were used: Baicalin (Chengdu Pufei De Biotech Co., Ltd., Chengdu, China; 96.81% purity), goat anti-bovine immunoglobulin G (IgG) heavy and light chains (H + L) (Medimmune, Gaithersburg, MD, USA; 20878), fetal bovine serum (FBS; HyClone, Logan, UT, USA), Dulbecco's modified Eagle's medium (DMEM)/high glucose (HyClone), donor equine serum (DES, HyClone), Cell Counting Kit-8 (CCK-8) cell proliferation and cytotoxicity assay kit (Suzhou Yuheng Biotech Co., Ltd., Suzhou, China), Madin-Darby bovine kidney cell line (MDBK; Shanghai Cell Cultures, Chinese Academy of Sciences, Shanghai, China), and BVDV serum (strain NADL, China Institute of Veterinary Drug Control). All other reagents were domestic and had analytical purity.

The protocol was approved by the Jilin Agricultural University Institutional Animal Care and Use Committee, Date of the approval: 2019.12.04.

Methods

Esterification process of baicalin ester derivatives and structural identification

Baicalin (222 mg, 0.5 mmol) was added to a 100 mL single-mouth vial, and 16 mL of methanol, ethanol, propanol, butanol, hexanol, or heptanol was added. Then, 300 µL of sulfoxide chloride was added dropwise at 0°C with stirring, followed by stirring at different temperatures for 14 h. The solvent was then evaporated under reduced pressure. Next, 20 mL of methanol, ethanol, propanol, butanol, hexanol, or heptanol was added, the precipitate dissolved, and the solvent was evaporated off again, before 20 mL of ether was added to each tube. The six baicalin ester compounds were obtained by filtration and measured using mass spectrometry (MS), electrospray ionization MS (ESI-MS), ^{1}H nuclear magnetic resonance (NMR), and ^{13}C NMR. Figure 1 shows the specific reaction flow.

Molecular docking program Auto Dock 4.2 prediction of the binding of baicalin derivatives and BVDV NS5B polymerase

The structure of BVDV NS5B polymerase was obtained from Protein Data Bank (PDB) (http://www.rcsb.org/pdb). ChemBio3D Ultra 11.0 software (Cambridge soft Corp., Waltham, MA, USA) was used to set the three-dimensional structure of the small molecule ligand. The binding of the ligands and BVDV NS5B was predicted using molecular docking with Auto Dock 4.2. The coordinate center position of the interface pocket was determined as X = 8.1795, Y = 33.8703, Z = 73.3317, and the size of the grid box was 80 × 80 × 80 (Å). The spacing of each grid point was 0.375 Å, and the docking results were visualized using PyMOL and LigPlotL.[29-31]

Molecular dynamics simulation studies

The molecular dynamics (MD) studies for baicalin ethyl ester and BVDV NS5B were performed for 50 ns using GROMACS version 5.1.4[35] employing the AMBER[36] force field. Na^{+} and Cl^{-} were added to the system to neutralize the electrical neutrality of the system and to establish a transferable intermolecular potential 3 point (TIP3P)[37] water model with a side length of 10 Å. The steepest descent algorithm[38] was used to minimize the energy, and then, the system was slowly ramped up from 0 to 300 K to equilibrate the solvent and ions around the protein. Then, a 50 ns MD simulation of the system was performed using a 2 fs time step, with traces saved every 10 ps.

Study of the antiviral effect of baicalin and its ester derivatives

Preparation of test sample solution

Twenty milligrams of baicalin and its derivatives was weighed accurately and added to a 2 mL volumetric flask. The compounds were dissolved in

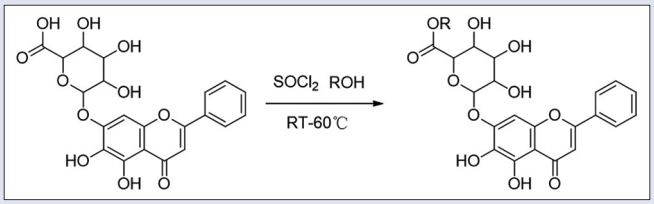

Figure 1: Flowchart of the reaction

2 mL of dimethyl sulfoxide (DMSO) solution to obtain a 10 mg/mL stock solution. The solutions were filtered through a sterile 0.22 μm filtration membrane and placed at 4°C. The solutions of baicalin and its derivatives were diluted to different concentrations using a twofold gradient dilution method with cell maintenance solution (containing 2% FBS) for use in subsequent experiments.

MDBK cell recovery and passage

MDBK cells were removed from liquid nitrogen, incubated at 37°C, centrifuged to remove the freezing solution, added to 8% bovine serum in DMEM, and placed in a 37°C incubator containing 5% CO_2. After the cells had grown into a monolayer, they were washed three times with 1 × phosphate-buffered saline (PBS), digested with 0.25% trypsin, centrifuged, and 8% bovine serum culture medium was added to continue culture in a 37°C, 5% CO_2 incubator.

In vitro MDBK cytotoxicity determination of baicalin and its ester derivative

When the cells were in the logarithmic growth phase, the density was adjusted to 2.0 × 10^5/mL using a cell counter and 100 μL of cells was added to a 96-well plate. After 12 h of incubation, the cells were washed three times with PBS and then different concentrations of drugs were added. Each group comprised six replicates, and a control group and a blank group were also set up. After 48 h, 10 μL of CCK-8 solution was added to each well and the OD_{450} value was determined after 1 h of incubation to calculate the cell survival rate and inhibition rate, which were used to determine the maximum safe concentration of the esters for the cells. The half-maximal inhibitory concentrations (IC_{50} values) were obtained from the regression equations derived from their quantitative-effect relationship plots for MDBK cells.

Viral titer determination

MDBK cells were seeded in 96-well plates and continued to grow for 12 h until 80% confluence was attained. To titrate the virus with a 50% tissue culture infective dose of virus ($TCID_{50}$), we infected cells at 80% confluence in 96-well plates with the virus at a series of dilutions from 10^{-1} to 10^{-10} with eight wells for each dilution. Then, the cells were maintained at 37°C in 5% CO_2 for 5 d. The titer of $TCID_{50}$ was determined with immunoperoxidase monolayer assay (IPMA).

Determination of anti-BVDV activity of baicalin and its ester derivatives in vitro

When the MDBK cells were in the logarithmic growth phase, the density was adjusted to 2.0 × 10^5/mL using a cell counter and the cells were added into a 96-well culture plate at 100 μL/well. When the cells had grown into a monolayer, they were washed three times with PBS, added to 100 μL of different drug solutions and 100 μL of 100 $TCID_{50}$ virus solution, and incubated for 4 h. The medium was then discarded, and different concentrations of baicalin and its ester derivative solution were added separately at a concentration of 200 μL/well. A virus group, a control group, and a blank group, and eight parallel wells were set up for each group of experiments. The cells were incubated for 3 d, and the cell growth status was observed daily and the cell cytopathic effect (CPE) was recorded. After 3 d, the 96-well culture plates were removed and incubated with 20 μL of CCK8 solution per well for 3 h. The OD_{450} values were measured, and the IC_{50} was calculated.

Table 1: Primers sequences

Name	Primer	Sequence (5′-3′)
BVDV	Forward	GGATGCCATGTGGACGAGGGCG
	Reverse	GCATGTGCCATGTACAGCAGAGG

BVDV=bovine viral diarrhea virus

Detection of baicalin and its ester derivatives on BVDV RNA levels by RT-PCR

Primer design and synthesis

Primer design and synthesis from BVDV were obtained from National Center for Biotechnology Information (NCBI) GenBank database. Forward primers and reverse primers were used for the amplification of 5′-untranslated region (UTR) of the BVDV genome using real-time Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Primers were designed with Primer Express 5.0 designer software and synthesized by Sangon Biotech (Shanghai, China). The primer sequences are shown in Table 1.

Create standard curves

In this study, 10-fold serial dilutions of RNA were prepared for the standard curve. The Ct value is the ordinate coordinate (Y) and the logarithm of RNA dilution is the horizontal coordinate (X). Real-time RT-PCR was used (One Step PremixScript™ RT-PCR kit; Takara Bio Inc., Beijing, China).

Real-time RT-PCR

When the MDBK cells were in the logarithmic growth phase, the density was adjusted to 2.0 × 10^5/mL using a cell counter and the cells were added into a six-well culture plate at 2 mL/well. When the cells had grown into a monolayer, they were washed three times with PBS, added to 2 mL of different drug solutions and 100 $TCID_{50}$ virus solution, and incubated for 4 h. The medium was then discarded and different concentrations of baicalin and its ester derivatives solution were added separately. After 3 d, cellular RNA was extracted and reverse transcribed into cDNA according to the instructions of the reverse transcription kit. The viral load was calculated according to the standard curve and the Ct value of each group, which was then used to determine the antiviral effect of the drugs.

Data analysis

Multiple replicates of each experiment were carried out, and the IC_{50} values of baicalin derivatives against MDBK were calculated by GraphPad Prism 7 (GraphPad Inc., La Jolla, CA, USA). One-way analysis of variance (ANOVA) followed by Newman–Keuls quantity analysis was used to assess the statistical relevance among the variable groups. The statistical difference was considered to be significant at the level of $P < 0.05$ (**).

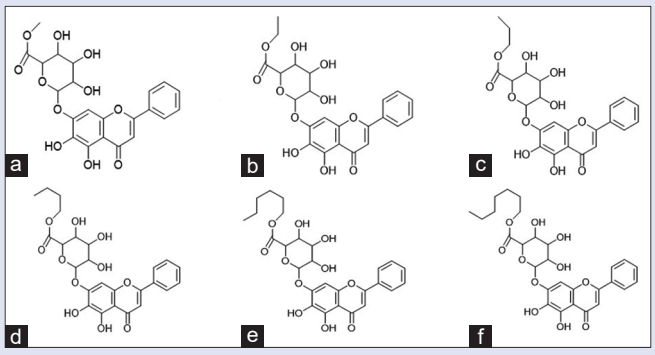

Figure 2: Structure of baicalin ester derivatives: (a) baicalin methyl ester, (b) baicalin ethyl ester, (c) baicalin propyl ester, (d) baicalin butyl ester, (e) baicalin hexyl ester, (f) baicalin heptyl ester

Table 2: Physical and chemical properties and MS data for baicalin derivatives

Compound	Appearance	mp (°C)	Yield (%)	ESI-MS, *m/z* [M-H]$^-$
Baicalin methyl ester	Yellow powder	170.9-173.8	80	459.0
Baicalin ethyl ester	Yellow powder	205.6-208.0	76	473.0
Baicalin propyl ester	Yellow powder	181.5-185.9	71	487.1
Baicalin butyl ester	Orange powder	215.6-218.4	77	501.0
Baicalin hexyl ester	Yellow powder	188.9-190.0	27	529.1
Baicalin heptyl ester	Yellow powder	202.0-204.0	65	543.1

ESI-MS=electrospray ionization mass spectrometry, mp=melting point, MS=mass spectrometry

Table 3: ^{1}H NMR and ^{13}C NMR for baicalin derivatives

Compound	^{1}H NMR (600 MHz) δ, ^{13}C NMR (150 MHz) δ	
Baicalin methyl ester	^{1}H NMR	12.59 (s, 1H, −OH), 8.68 (s, 1H, −OH), 8.08 (d, 2H), 7.61 (d, 3H), 7.06 (s, 1H), 7.01 (s, 1H), 5.52 (q, 2H), 5.31 (q, 2H), 4.22 (d, 1H), 3.67 (s, 3H), 3.43 (s, 2H), 3.36 (s, 1H)
	^{13}C NMR	182.5, 169.2, 163.5, 151.2, 149.2, 146.8, 132.0, 130.8, 130.6, 129.1, 129.1, 126.4, 126.4, 106.1, 104.8, 99.8, 93.6, 75.2, 75.0, 72.7, 71.4, 52.0
Baicalin ethyl ester	^{1}H NMR	12.58 (s, 1H, −OH), 8.68 (s, 1H, −OH), 8.07 (d, 2H), 7.60 (d, 3H), 7.06 (s, 1H), 7.01 (s, 1H), 5.52 (d, 1H), 5.48 (d, 1H), 5.30 (q, 2H), 4.18 (d, 1H), 4.15 (s, 2H), 3.45 (q, 2H), 3.32-3.39 (m, 1H), 1.21 (s, 3H)
	^{13}C NMR	182.5, 168.6, 163.5, 151.2, 149.1, 146.8, 132.0, 130.8, 130.6, 129.1, 129.1, 126.3, 126.3, 106.1, 104.8, 100.0, 93.7, 75.3, 75.1, 72.8, 71.3, 60.7, 13.9
Baicalin propyl ester	^{1}H NMR	12.57 (s, 1H), 8.68 (s, 1H), 8.06 (d, 2H), 7.60 (d, 3H), 7.04 (s, 1H), 7.01 (s, 1H), 5.27 (d, 1H), 4.20 (d, 1H), 4.08 (s, 1H), 4.04 (s, 1H), 3.37-3.47 (m, 6H), 1.60 (d, 2H), 0.88 (t, 3H)
	^{13}C NMR	182.5, 168.6, 163.5, 151.2, 149.1, 146.8, 132.1, 130.8, 130.6, 129.1, 129.1, 126.3, 126.3, 106.1, 104.8, 100.1, 93.8, 75.3, 75.1, 72.8, 71.2, 66.1, 21.4, 10.1
Baicalin butyl ester	^{1}H NMR	12.57 (s, 1H), 8.68 (s, 1H), 8.06 (t, 2H), 7.60 (d, 3H), 7.03 (s, 1H), 7.01 (s, 1H), 5.28 (d, 1H), 4.19 (d, 1H), 4.12 (s, 1H), 4.07 (s, H), 3.36-3.47 (m, 6H), 1.55 (d, 2H), 1.31 (q, 2H), 0.80 (t, 3H)
	^{13}C NMR	182.5, 168.6, 163.5, 151.2, 149.1, 146.8, 132.0, 130.8, 130.6, 129.1, 129.1, 126.3, 126.3, 106.1, 104.8, 100.0, 93.8, 75.3, 75.1, 72.8, 71.2, 64.3, 30.0, 18.4, 13.4
Baicalin hexyl ester	^{1}H NMR	12.57 (s, 1H), 8.68 (s, 1H), 8.06 (t, 2H), 7.59 (d, 3H), 7.03 (s, 1H), 7.01 (s, 1H), 5.28 (d, 1H), 4.19 (d, 1H), 4.12 (s, 1H), 4.06 (s, 1H), 3.37–3.47 (m, 6H), 1.55 (t, 2H), 1.39 (d, 4H), 1.13 (d, 2H), 0.71 (s, 3H)
	^{13}C NMR	182.5, 168.6, 163.5, 151.2, 149.1, 46.7, 132.0, 130.8, 130.6, 129.1, 129.1, 126.3, 126.3, 106.1, 104.7, 99.9, 93.7, 75.2, 75.1, 72.8, 71.1, 64.5, 30.7, 27.9, 24.8, 21.8, 13.7
Baicalin heptyl ester	^{1}H NMR	12.57 (s, 1H), 8.68 (s, 1H), 8.06 (d, 2H), 7.60 (d, 3H), 7.02 (d, 2H), 5.28 (d, 1H), 4.19 (d, 1H), 4.13 (s, 1H), 4.05 (s, 1H), 3.35–3.47 (m, 6H), 1.55 (t, 2H), 1.24 (s, 6H), 1.09 (s, 2H), 0.72 (t, 3H)
	^{13}C NMR	182.5, 168.5, 163.5, 151.2, 149.1, 146.7, 132.0, 130.8, 130.6, 129.1, 129.1, 126.3, 126.3, 106.1, 104.7, 100.0, 93.7, 75.2, 75.1, 72.8, 71.1, 64.5, 31.1, 28.2, 28.0, 25.1, 21.9, 13.8

NMR=nuclear magnetic resonance

RESULTS

Structure identification results of baicalin derivatives

The structures of the baicalin ester derivatives are shown in Figure 2. The physico-chemical properties and characterization data of the baicalin derivatives are listed in Tables 2 and 3, respectively.

Predicting the binding of baicalin derivatives and BVDV NS5B polymerase using a molecular docking procedure

BVDV NS5B is situated at the protein's carboxyl terminus and contains the amino acid motif Gly-Asp-Asp,[39] which has a spatial conformation resembling a human right hand, and the whole can be divided into three parts comprising the finger, thumb, and palm. A segment of the loop region of its finger region extends above the thumb region and is interconnected with the thumb region amino acids through intermolecular forces, such as hydrophobic interactions, so that the entire NS5B presents a shape similar to the gesture for "OK." This special loop region (fingertip) is very important for the maintenance of the overall conformation of NS5B, and its location is one of the variable conformation sites (Thumb Site I) of the NS5B thumb region.[40] The binding pattern of baicalin ethyl ester to the NS5B Thumb Site I is clearly shown in the molecular docking results [Figure 3]. Four hydrogen bonds formed between baicalin ethyl ester and NS5B can be clearly seen in the figure. The hydroxyl group of the A-ring *o*-diphenol of baicalin ethyl ester and the hydrogen atom in residue Gln127 form the first hydrogen bond (bond length = 3.05 Å); the second and third hydrogen bonds are formed by the hydroxyl and oxygen atoms of 2H-pyran and the hydrogen atoms of residues Trp187 and Asn186 (bond lengths = 3.34 Å and 2.92 Å, respectively), and the other hydrogen bond is formed by the 4-position hydroxyl group of glucuronide and the carbonyl oxygen atom in residue Glu100 (bond length = 2.65 Å), which made ethyl baicalin more stable in the presence of NS5B polymerase [Figure 3]. The other derivatives and baicalin ethyl ester share the same backbone and their active conformations are similar; therefore, the formation of hydrogen bonds with two residues, Asn186 and Glu100, is a common feature of this group of baicalin glucuronides.

The binding pattern of baicalin and its derivatives to BVDV NS5B is clearly shown in Figure 3A–G. Comparison of baicalin [Figure 3G] with baicalin methyl ester [Figure 3B] shows that the Bring of the baicalin parent nucleus does not fully penetrate into the hydrophobic pocket formed by residues Gly167, Glu100, Cys168, and Cys130, resulting in the absence of hydrogen bond formation of baicalin, and therefore, the binding energy of baicalin is not as high as the binding energy of baicalin methyl ester. On comparing baicalin methyl ester [Figure 3B] and baicalin ethyl ester [Figure 3A], it can be seen that the binding energy of baicalin ethyl ester is better than that of baicalin methyl ester, because baicalin ethyl ester has one more carbon than baicalin methyl ester, the spatial conformation is changed, and the hydrogen bond is

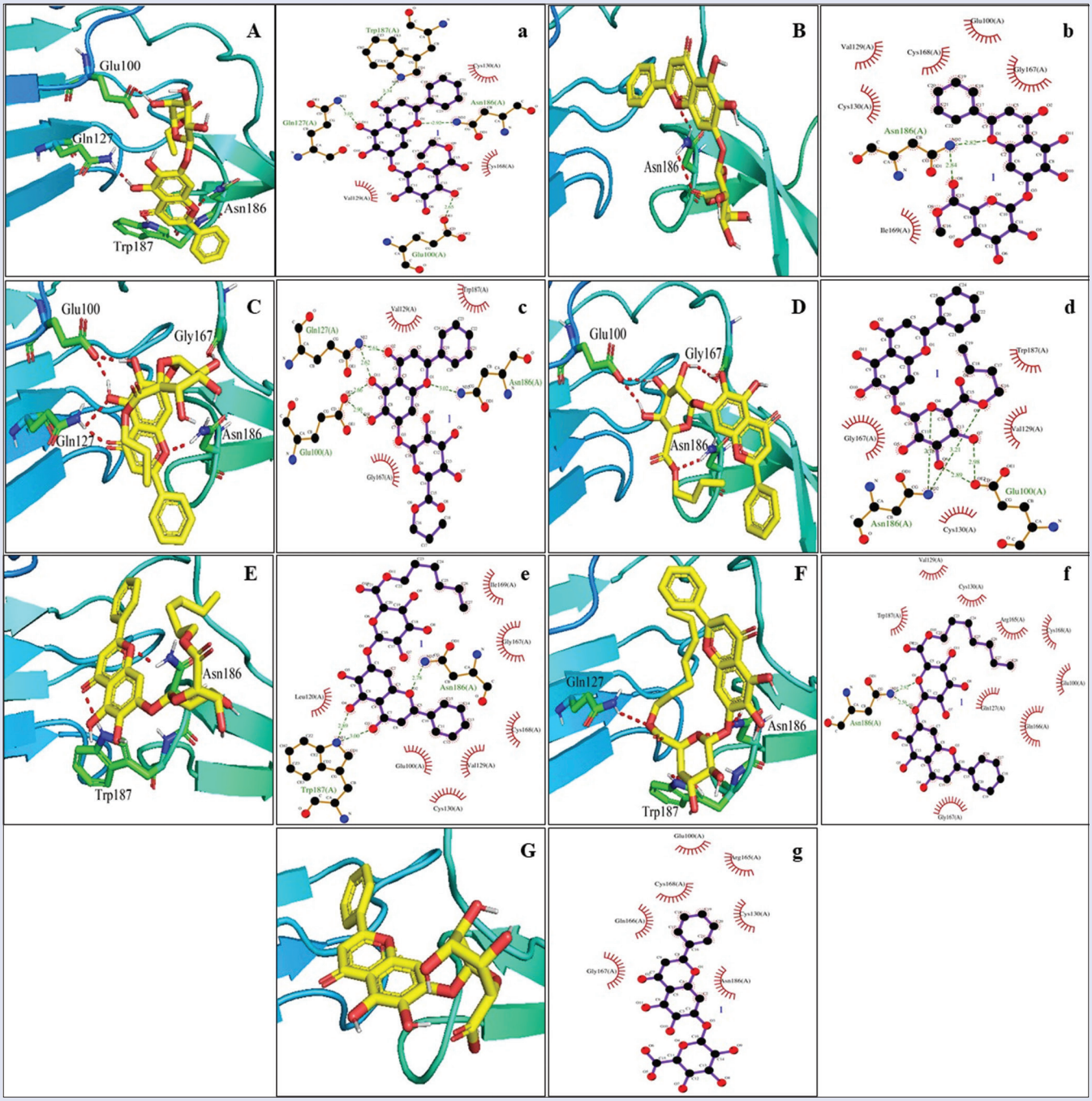

Figure 3: Docking map of baicalin and its ester derivatives with BVDV NS5B. A-a, B-b, C-c, D-d, E-e, F-f, and G-g represent separate docking of baicalin ethyl ester, baicalin methyl ester, baicalin propyl ester, baicalin butyl ester, baicalin hexyl ester, baicalin heptyl ester, and baicalin with Thumb Site I of BVDV NS5B polymerase, respectively. BVDV = bovine viral diarrhea virus, NS5B = non-structural protein 5B

formed between the 4position hydroxyl group of glucuronide and the carbonyl oxygen atom in residue Glu100, which makes baicalin ethyl ester more stable in the presence of NS5B. Interestingly, comparison of baicalin ethyl ester [Figure 3A] and baicalin propyl ester with baicalin heptyl ester [Figure 3C–F] showed that although these baicalin analogs have similar spatial conformations, the terminal spatial conformation of the compound is changed because of the stepwise increase of carbon at its glucuronide end. They cannot fully penetrate into the hydrophobic pocket, and the number of hydrogen bonds decreases proportionately and the bond length of the hydrogen bonds with the residues is gradually extended. Therefore, compared with baicalin ethyl ester, the binding energy values of baicalin propyl ester and baicalin heptyl ester to NS5B are reduced. The binding energy values of each compound with BVDV NS5B are shown in Table 4.

Molecular simulation study

Root mean square deviation (RMSD) can describe the size of the change in conformation after simulation and can be used to estimate the stability of protein and ligand complexes. Figure 4a shows the RMSD values of BVDV NS5B and baicalin ethyl ester during the 50 ns simulation.

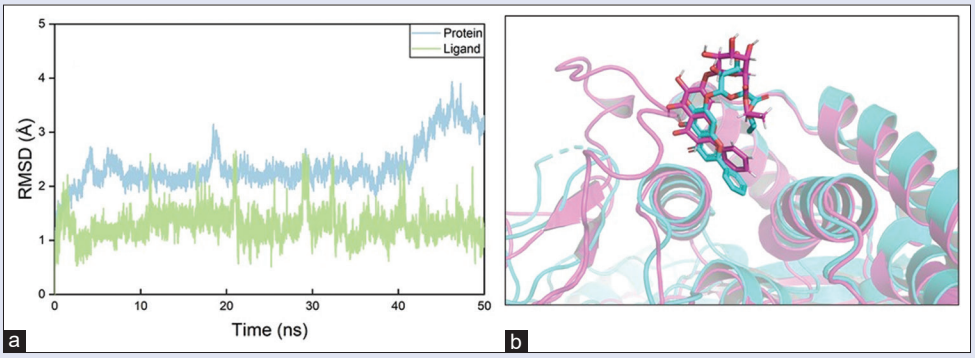

Figure 4: Simulation of a complex system: (a) RMSD, (b) conformational changes during MD, cyan is the docking result, magenta is the last frame of the track. MD = molecular docking, RMSD = root mean square deviation

Baicalin ethyl ester showed deviations during the initial simulations, but over time, it was stable at around an RMSD value of 1 Å, indicating excellent stability of the docking model. Figure 4b shows the docking results overlaid with the last frame of the MD simulation, which shows that the ligand remains in the pocket and the binding pattern is similar to that of the docking results. The structural superimposition also showed no significant conformational changes in the complex as a whole during the simulations, once again demonstrating the good stability of the docking model and further proving the reliability of the docking results.

Antiviral activity study of baicalin and its ester derivatives

Cytotoxicity of baicalin and ester derivatives

The IC_{50} values of the different drugs on MDBK cell proliferation in the concentration range of 200–1.565 μg/mL are shown in Table 4, which were used to determine a reasonable assay concentration for the antiviral activity of each compound. None of the compounds inhibited MDBK cell proliferation and they did not affect normal cell morphology at the concentration tested for antiviral activity.

BVDV virulence determination

The virulence of BVDV was determined by IPMA. The cells developed lesions after BVDV inoculation, with obvious shedding and agglomeration, and the shape became round. Compared with the control group, the refraction index was poor. After 3-Amino-9-Ethylcarbazole (AEC) color development, the virus-infected cells (CPE) were stained reddish-brown, as shown in Figure 5, and the negative control group did not produce reddish-brown color. The number of cells with positive color was counted, and the virulence of BVDV was calculated as $10^{4.75}$/0.1 mL according to the Karber formula lgTC ID_{50} = L − d (s − 0.5) [Table 5].

Inhibition of BVDV replication by baicalin and ester derivatives *in vitro*

The OD_{450} value measured by the CCK-8 assay was used to calculate the antiviral efficiency of the drug, and the quantitative-effect relationship between the logarithm of the drug concentration (X) and the antiviral efficiency of the drug (Y) was established. The IC_{50} was calculated using a regression equation, as shown in Table 6. Both baicalin and its ester derivatives showed significant inhibitory effects on BVDV replication *in vitro*, and cytopathic conditions were observed after the drug action on the virus, with less cell shedding and clustering in the drug administration group than in the viral group. Whether the highest antiviral efficiency or the IC_{50} was used as an indicator, the inhibitory effects of baicalin and its ester derivatives on BVDV replication were basically in the same order.

Table 4: The IC_{50} values of target compounds on MDBK cells

Compound	Regression equation	IC_{50} (μg/mL)
Baicalin	Y=71.06X-66.55	45.29
Baicalin methyl ester	Y=57.95X-54.57	63.75
Baicalin ethyl ester	Y=39.48X-26.13	84.72
Baicalin propyl ester	Y=31.45X-19.21	138.48
Baicalin butyl ester	Y=34.81X-24.36	126.77
Baicalin hexyl ester	Y=40.80X-20.82	58.36
Baicalin heptyl ester	Y=59.06X-82.25	176.54

IC_{50}=half-maximal inhibitory concentration, MDBK=Madin-Darby bovine kidney cells

Table 5: BVDV vaccination of cells with different dilutions

Virus dilution	Inoculation hole	No CPE hole	CPE hole	CPE ratio	CPE (%)
10^{-1}	8	0	8	8/8	100
10^{-2}	8	1	7	7/8	87.5
10^{-3}	8	3	5	5/8	62.5
10^{-4}	8	4	4	4/8	50.0
10^{-5}	8	4	4	4/8	50.0
10^{-6}	8	5	3	3/8	37.5
10^{-7}	8	6	2	2/8	25
10^{-8}	8	7	1	1/8	12.5

BVDV = bovine viral diarrhea virus, CPE = cytopathic effect

Standard curves

The standard curve is Y = −1.3881X + 35.769 (R^2 = 0.9916). Y represents the Ct value and X represents the logarithm of RNA dilution, as shown in Figure 6.

The effects of the compounds on the viral loads

The effects of baicalin and its ester derivatives on viral loads were examined by real-time RT-PCR. Compared with the virus control group, treatment with baicalin and its ester derivatives astonishingly ($P < 0.001$) reduced the viral load [Figure 7]. The RNA content of BVDV in baicalin and its ester derivatives ranged from high to low as follows: baicalin ethyl ester > baicalin propyl ester > baicalin butyl ester > baicalin methyl ester > baicalin hexyl ester > baicalin heptyl ester > baicalin. Among them, baicalin ethyl ester had the best inhibitory effect on BVDV and the viral load was only 7.10%. The results of molecular docking were

Table 6: The inhibitory effects of target compounds against BVDV replication

Compound	Regression equation	Maximum antiviral efficiency of BVDV (%)	IC_{50} (μg/mL)	Binding energy
Baicalin	Y=16.67X+32.73	50.46	20.38	−0.37
Baicalin methyl ester	Y=23.94X+35.12	63.12	4.71	−1.41
Baicalin ethyl ester	Y=27.99X+33.95	73.33	3.08	−2.89
Baicalin propyl ester	Y=28.24X+36.68	71.46	4.28	−2.09
Baicalin butyl ester	Y=28.79X+22.25	63.78	8.53	−1.46
Baicalin hexyl ester	Y=22.02X+27.88	57.93	10.30	0.61
Baicalin heptyl ester	Y=29.35X+11.72	52.60	20.18	−0.95

BVDV=bovine viral diarrhea virus, IC_{50}=half-maximal inhibitory concentration

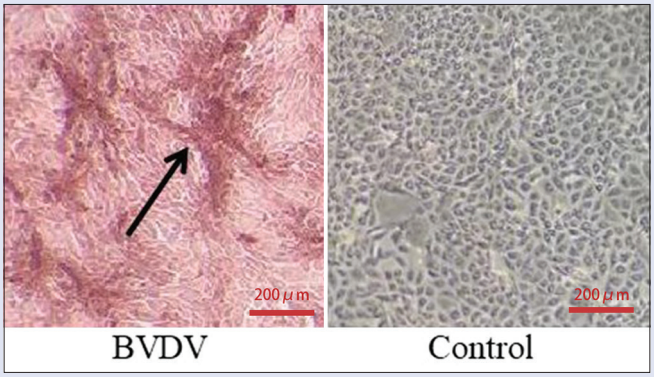

Figure 5: BVDV $TCID_{50}$ determination by IPMA. BVDV = bovine viral diarrhea virus, IPMA = immunoperoxidase monolayer assay, $TCID_{50}$ = 50% tissue culture infective dose of virus

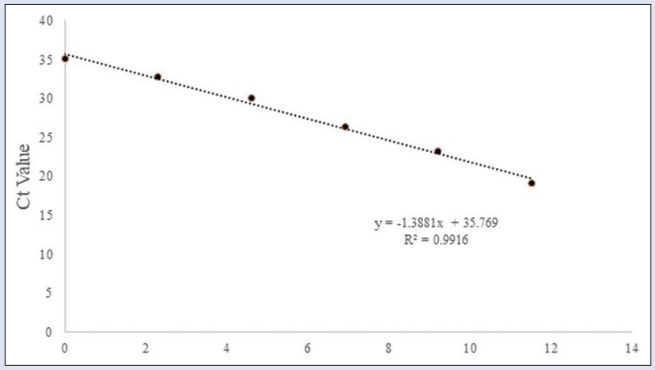

Figure 6: Standard curves

well verified by CCK-8 and real-time fluorescence quantitative PCR *in vitro*.

DISCUSSION

BVDV is a viral infectious disease that mainly infects cattle. In 2012, the Office international des épizooties (OIE) classified BVDV as a category B animal disease. BVDV can infect not only cattle, but also pigs, goats, sheep, deer, and other species.[41] The main route of transmission of BVDV is through direct contact between animals,[42] and the infected animals can transmit BVDV through excreta, semen, urine, saliva, and so on.[43] Infection with BVDV can cause a variety of clinical reactions, including respiratory and gastrointestinal diseases of varying severity, decreased fertility and calving rates in reproduction, which can seriously lead to miscarriage and fetal malformation in pregnant cattle.[44,45] In the campaign for the control and eradication of BVDV, countries such as Finland,[46] Sweden,[47] Norway,[48] Denmark,[49,50] and Austria[51] successfully got rid of BVDV without using vaccines through long-term monitoring of cattle. However, due to the high BVDV infection rate in Germany, Scotland, Belgium, and Ireland, vaccination has to be used as an additional control tool.[52] The vaccines of BVDV mainly include killed, live attenuated, and recombinant vaccines.[52-56] Live attenuated vaccines are widely used against BVDV by by triggering humoral and cell-mediated immune responses, but live attenuated vaccines based on NCP BVDV are not recommended for use in pregnant animals because NCP BVDV can infect fetuses through the placenta.[57-59] Although vaccination has a certain effect on the control of BVDV, some studies have shown that vaccination has not significantly reduced the incidence of BVDV in cattle populations.[60]

Traditional Chinese medicine has been used in China for thousands of years. It has become a research hotspot in academia due to its stable source, high cure rate, and few sequelae. Baicalin is the active representative component of traditional Chinese medicine *Scutellaria bailensis* Georgi. It has been found to be effective in the treatment of viral diseases, cancer,[61-63] inflammatory diseases,[7,9,64] and so on. Among the antiviral effects, baicalin was found to inhibit neuraminidase activity and was effective against influenza A strains H1N1 and H3N2.[21] In addition, baicalin, as a non-nucleoside reverse transcriptase inhibitor, is an active anti-HIV-1 drug that prevents HIV from entering the animal cells by interfering with the interaction of HIV-1 Env and HIV-1 co-receptors on the cell surface.[65]

Studies have found that baicalin has poor water solubility, fat solubility, and is easily decomposed by the intestinal flora, resulting in low bioavailability *in vivo*, which greatly limits the efficacy of baicalin.[66] Esterification reactions have been reported to increase the lipophilicity of flavonoids and improve the transmembrane permeability of the drug, thereby increasing the bioavailability of the drug.[33,34] Therefore, in this study, we chose to perform the esterification reaction on the carboxyl group at the 6-position of 7-β-d-glucuronide in the structure of baicalin. By controlling the reaction temperature, we finally obtained six compounds: baicalin methyl ester, baicalin ethyl ester, baicalin propyl ester, baicalin butyl ester, baicalin hexyl ester, and baicalin heptyl ester.

BVDV encodes 12 proteins, which are Npro, C, Erns, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B from N-terminal to C-terminal. NS5B is a non-structural protein located at the carboxyl terminus of the polyprotein, which is highly conserved in pestiviruses. NS5B plays a key role in transcription and replication in the BVDV genome.[6] Therefore, this study used molecular docking and MD to simulate the effect of baicalin and its ester derivatives on BVDV NS5B, in order to investigate its inhibitory effect on BVDV. The results showed that the binding energies of the seven drugs to BVDV NS5B were different and the binding energy of baicalin was lower than that of the other six ester derivatives. This is due to the change in the polarity of baicalin due to the introduction of alkanes, thereby enhancing the binding ability to BVDV NS5B. Duan *et al.*[67] found that newly developed nanobody can significantly reduce the replication of BVDV and

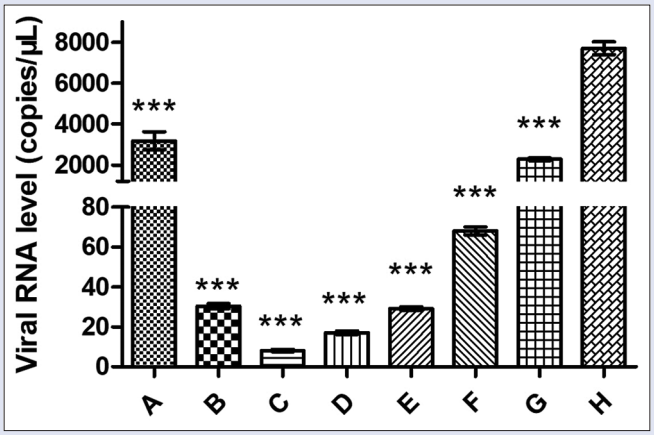

Figure 7: Compared with BVDV group: ***$P < 0.001$. BVDV RNA levels from the released virus in the whole cell culture on treatment with different drugs: (A) baicalin, (B) baicalin methyl ester, (C) baicalin ethyl ester, (D) baicalin propyl ester, (E) baicalin butyl ester, (F) baicalin hexyl ester, (G) baicalin heptyl ester, (H) BVDV

interact with BVDV NS5B. Therefore, it is shown that BVDV NS5B can be used as a new target for the development of drugs to inhibit BVDV.

To further validate the effect of baicalin and its ester derivatives on BVDV, CCK-8 and real-time RT-PCR were used for the antiviral activity assay against BVDV. Surprisingly, *in vitro*, the results of CCK-8 and real-time RT-PCR were consistent with the results of molecular docking and baicalin ethyl ester had the best inhibitory effect on BVDV.

BVDV is an RNA virus that has a highly variable genome, but the functional regions of the non-structural proteins located in the cytoplasm are relatively conserved.[68] BVDV NS5B is a non-structural protein, which is highly conserved among pestiviruses. Therefore, this experiment combined single compounds of Chinese herbs with BVDV NS5B to explore whether anti-BVDV drugs can be developed through the NS5B target. At the same time, both CCK-8 and real-time RT-PCR methods were used to validate *in vitro* experiments. In addition, the limitation of this study is that baicalin and its ester derivatives were not studied with NS5B genes and proteins by real-time RT-PCR and western blot *in vitro*. However, this study provides a scientific reference for the further development and utilization of baicalin and the development of new anti-BVDV drugs.

CONCLUSION

In this study, baicalin methyl ester, baicalin ethyl ester, baicalin propyl ester, baicalin butyl ester, baicalin hexyl ester, and baicalin heptyl ester were obtained by esterification reaction on the carboxyl group at the 6-position of 7-β-d-glucuronide in the structure of baicalin. Baicalin and its ester derivatives were shown by molecular docking and *in vitro* experiments to exert anti-BVDV replication effects by targeting BVDV NS5B polymerase, and there is a certain relationship between the structure of the baicalin derivatives and their antiviral activity.

Authors contributions

Ying Zong is responsible for conception, design, literature search, data acquisition, statistical analysis, manuscript preparation, and manuscript editing. Yuhan Sun is responsible for design, literature search, experimental studies, data acquisition, data analysis, manuscript preparation, and manuscript editing. The first two authors contributed equally to this work and are considered co-first authors. Yi Che is responsible for the conception and definition of intellectual content. Hui Wang is responsible for the design and experimental studies. Kun Shi is responsible for the conception and experimental studies. Zhongmei He is responsible for the design and manuscript review. Rui Du is responsible for the conception and is a guarantor.

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Conflicts of interest

There are no conflicts of interest.

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