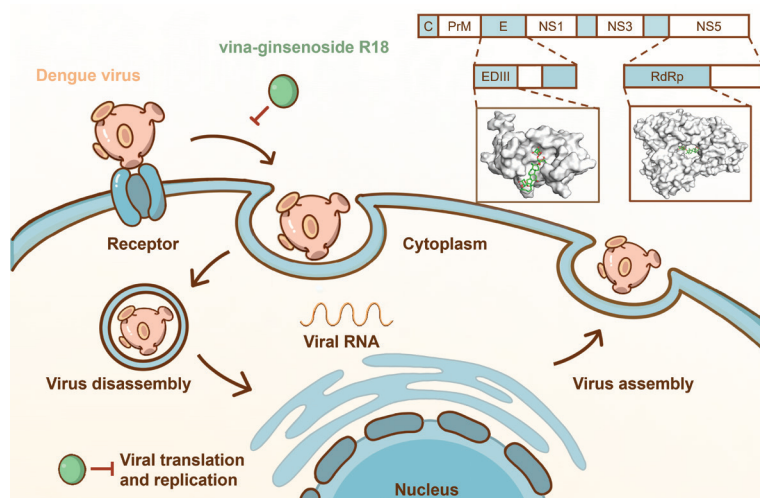


Discovery of vina-ginsenoside R18 as a novel dual-target inhibitor of dengue virus envelope protein and RNA-dependent RNA polymerase

Graphical abstract



Highlights

- Vina-ginsenoside R18 (R18) is a novel dual-target antiviral inhibitor of E protein and RdRp against dengue virus *in vitro* and *in vivo*.
- R18 disrupts the interaction of E protein with $\beta 3$ integrin to prevent viral adsorption to the host cells.
- R18 induces a conformational change of RdRp, leading to a decrease in its enzyme activity.

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In brief

The natural product vina-ginsenoside R18 acts as a novel dual-target antiviral against dengue virus. It inhibits viral adsorption by binding to envelope protein domain III and suppresses genome replication by altering RdRp conformation, ultimately alleviating infection and disease symptoms.

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Discovery of vina-ginsenoside R18 as a novel dual-target inhibitor of dengue virus envelope protein and RNA-dependent RNA polymerase

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Received: 23 December 2024; Revised: 30 April 2025; Accepted: 29 September 2025

Published online: 1 November 2025

DOI 10.15212/AMM-2024-0094

ABSTRACT

Dengue virus (DENV) poses a serious health threat globally for which specific antivirals are not available. Envelope (E) protein and RNA-dependent RNA polymerase (RdRp) from DENV represent the most critical targets for the development of antivirals that inhibit viral adsorption for entry and genome replication, respectively; no dual inhibitors targeting E/RdRp have been reported to date. Vina-ginsenoside R18 (R18), a triterpenoid saponin isolated from *Panax notoginseng*, was shown to target E protein domain III to inhibit binding of E protein to integrin beta3, thus preventing viral adsorption to the host surface receptor. R18 selectively targets RdRp protein and induces a conformational change, leading to a decrease in enzyme activity. Consequently, R18 represses DENV infection by acting at viral binding, entry, and replication stages, and attenuates the pathologic symptoms of DENV-infected ICR suckling and AG129 mice. These results showed R18 to be a novel dual E/RdRp inhibitor that can serve a potential agent against DENV infection.

Keywords: Dengue virus, Envelope protein, RNA-dependent RNA polymerase, Target, Inhibitor

1. INTRODUCTION

Dengue virus (DENV) is a member of the *Flaviviridae* family and one of the most prevalent mosquito-borne viruses worldwide [1]. DENV is classified by serotype (DENV-1–4) based on antigenic differences. The incidence of dengue has grown dramatically in recent decades with an estimated range of 100–400 million infections and 500,000–1,000,000 cases progressing to severe dengue, which ultimately leads to approximately 25,000 deaths [2–4]. Currently, there is a lack of therapeutic drugs against DENV. The first approved dengue vaccine, Dengvaxia, has been shown to exhibit an unsatisfactory protective response against DENV, especially against the most prevalent serotype, DENV-2.

Furthermore, seronegative individuals may be more susceptible to severe dengue from this vaccine due to the presence of antibody-dependent enhancement (ADE) of infection, which restricts use of the vaccine [5, 6]. QDENG A was approved in 2022 for individuals ≥ 4 years of age but has not demonstrated efficacy against DENV-3 in seronegative individuals and efficacy data for DENV-4 are inconclusive [7].

The DENV genome encodes a polyprotein, which is cleaved by proteolysis and translates three structural proteins (capsid C, precursor membrane prM or membrane M, and envelope E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [8]. E protein is the main glycoprotein and is crucial to the pathogenesis underlying DENV and immune response

against DENV. E protein is mainly composed of three distinct domains (ED I, ED II, and ED III), of which ED III has a structure similar to an immunoglobulin that mediates binding of DENV to cell surface receptors, hence enabling DENV entry into the host cell [9–11]. In addition, the NS5 protein is the largest and most conserved protein with an RNA-dependent RNA polymerase (RdRp) at the C-terminus and a methyltransferase (MTase) at the N-terminus. RdRp is directly involved in the synthesis of DENV genomic RNA and is essential for viral genome replication and transcription [12, 13]. The important role of E protein and RdRp in the DENV replication cycle makes the E protein and RdRp appealing targets for the development of inhibitors against DENV.

Direct-acting antivirals (DAAs) are a promising class of compounds that act by directly targeting viral proteins and may cause fewer side effects and less risk of off-target effects than host-directed antivirals (HDAs) [14]. DAAs inhibit viral infection by acting at stages of the viral life cycle [15]. Blocking viral entry and inhibiting viral enzyme activity have both proven to be successful antiviral strategies. However, viral gene sequences are highly prone to mutations, which often leads to the emergence of DAA resistance. In contrast, dual-target inhibitors achieve synergistic antiviral effects and a significantly elevated genetic barrier to resistance by acting on multiple stages of the viral life cycle or on both viral and host proteins. These advantages have shifted our research focus toward dual-target inhibitors. Examples of antiviral compounds with dual mechanisms of action have been reported previously. A representative drug is suramine, an antiparasitic agent, that not only blocks human immunodeficiency virus (HIV) entry by binding to the envelope glycoprotein (gp120) but also suppresses reverse transcriptase activity [16, 17]. Another compound that affects both HIV entry and reverse transcriptase activity is epigallocatechin gallate [18, 19]. Among flaviviruses, emetine has been proposed to disrupt zika virus (ZIKV) entry and activity of the polymerase [20]. Currently, many DAAs targeting the DENV E protein or RdRp have been discovered. NITD-448 directly targets the hydrophobic β OG pocket of E protein, which inhibits E protein-mediated membrane fusion and thus prevents viral entry into cells [21]. Balapiravir, a nucleotide analogue inhibitor of the hepatitis C virus (HCV) RdRp, is also effective against DENV due to the structural similarity of the RdRps [22]. However, no dual-target DAAs targeting both E and RdRp to inhibit DENV have been reported to date. Indeed, N-methylcytosine Thio derivatives have been shown to exhibit anti-dengue activity by targeting viral E protein and NS2B-NS3 protease [23].

In the current study vira-ginsenoside R18 (R18), a triterpenoid saponin isolated from the root of *Panax notoginseng* (Burk.) F. H. Chen, was shown to simultaneously target E protein and RdRp, and is the first identified dual E/RdRp inhibitor against DENV. Specifically, R18 competed with the host cell receptor, integrin β 3 (ITG β 3), for binding to ED III, which disrupted

the ED III-ITG β 3 interaction and ultimately prevented viral entry into cells. R18 also regulated conformation of RdRp to inhibit RdRp activity. This inhibition led to the protective effect of R18 against DENV-induced cell death and symptoms of ICR suckling and AG129 mice. Taken together, this study revealed that R18 may serve as a novel dual E/RdRp inhibitor for developing an antiviral drug against DENV and indicates that triterpenoid saponins may be a new source for discovering new compounds to combat DENV infection.

2. MATERIALS AND METHODS

2.1 Cell culture

Baby hamster kidney fibroblast (BHK-21) and *Aedes albopictus* cells (C6/36) were cultured in RPMI-1640 medium containing 10% fetal bovine serum [FBS] (ExCell Bio, Shanghai, China) at 37°C and 28°C, respectively. Human hepatocellular carcinoma Huh7, human non-small cell lung cancer A549, and African green monkey kidney Vero cells were cultured in DMEM medium supplemented with 10% FBS at 37°C.

2.2 Viruses

The DENV-2 New Guinea C (NGC) strain was kindly provided by Professor Xingang Yao (School of Pharmaceutical Sciences, Southern Medical University, Guangzhou, China). DENV-3 strain H87 was stored in the Guangdong Provincial Key Laboratory of Tropical Disease Research (Guangzhou, China).

DENV was propagated in C6/36 cells. Briefly, C6/36 cells were infected with DENV for 1 h at 37°C and incubated in RPMI-1640 medium containing 2% FBS. The supernatant viral solution was collected after the cells had fully developed cytopathic effects (CPEs). The viral titers were detected using the TCID₅₀ method and virus was stored at -80°C.

2.3 Chemicals

Fourteen dammarane-type triterpenoid saponins were isolated as previously reported [24]; the chemical structures are depicted in Figure 1A. The purity of R18 was analyzed by high-performance liquid chromatography (HPLC) and shown to be > 95% (Figure S1).

2.4 Recombinant protein

Recombinant ED III protein was kindly gifted by Professor Wei Zhao (Southern Medical University). Recombinant DENV-2 RdRp with a 6 × His-tag fused to the C-terminus was cloned into pET28a vector. The plasmid was transformed into BL21 (DE3) and cultured in LB medium with kanamycin overnight (250 rpm at 37°C). Next, 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) was supplemented to induce RdRp expression, followed by incubation at 20°C with shaking at 250 rpm for 12 h. The cell pellet was obtained by centrifugation and resuspended in lysis buffer [50 mM phosphate-buffered saline (PBS) {pH 7.4} and 0.15 M NaCl]. The resuspended cells were

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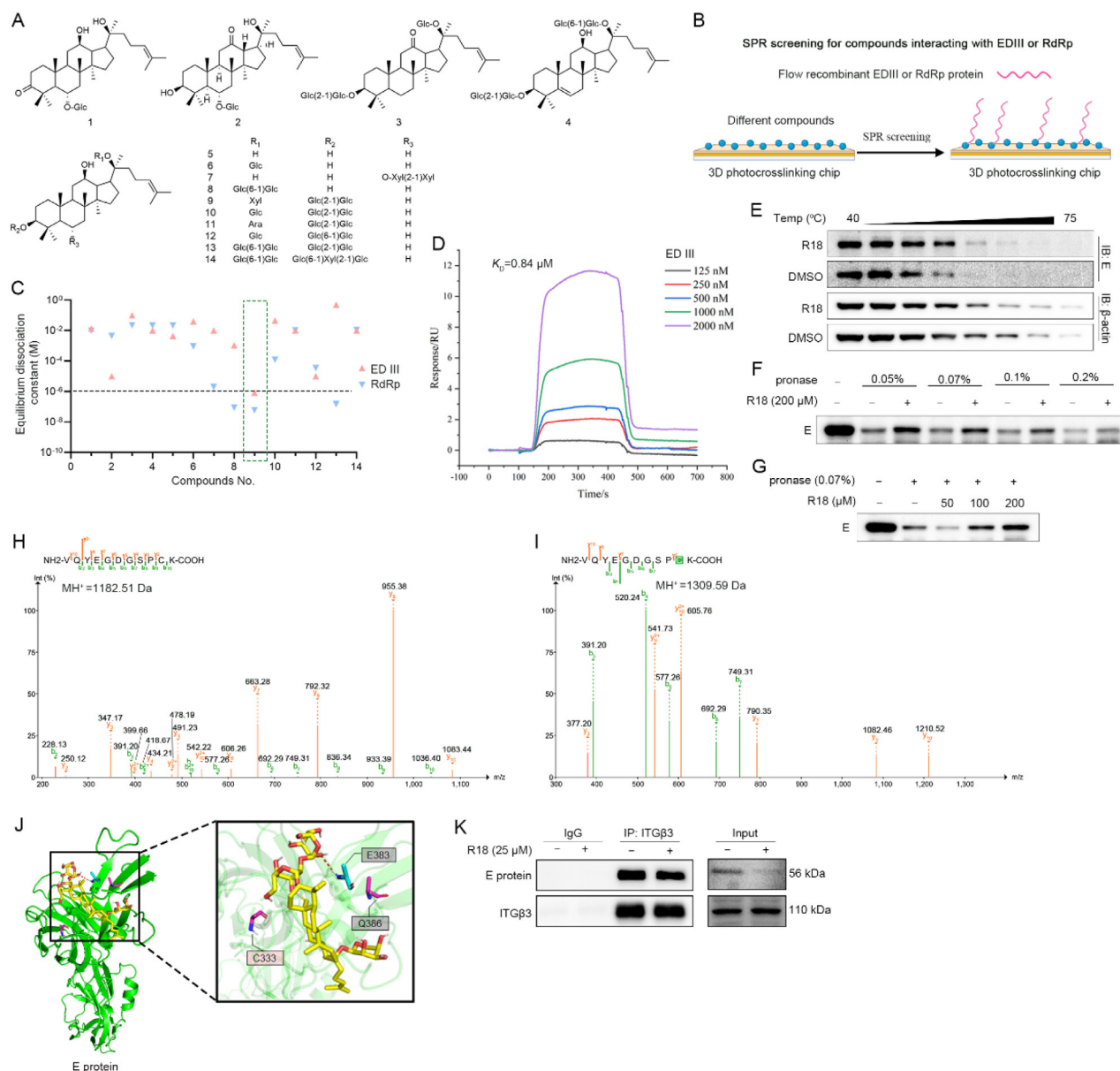


Figure 1 | R18 competitively binds E protein with ITGβ3.

(A) Chemical structures of 14 dammarane-type triterpenoid saponins. (B) Diagram of SPR screening for small molecules that interact with ED III or RdRp. (C) The equilibrium dissociation constants of 14 dammarane-type triterpenoid saponins with ED III or RdRp. (D) The binding affinity of R18 to ED III protein detected by SPR analysis. (E) R18 enhances the thermal stability of E protein. BHK-21 cells were infected with DENV-2 and the cell lysates were incubated with R18 or DMSO for CETSA. (F, G) R18 decreases the susceptibility of E protein to pronase. The DENV-2-infected BHK-21 cell lysates were incubated with R18 followed by the DARTS assay. (H, I) R18 covalently modifies E protein at residue C333. Recombinant E protein was incubated with R18 for 12 h, followed by SDS-PAGE and LC-MS/MS assays. (J) Molecular docking diagram of R18 and ED III protein. (K) R18 attenuates E protein interaction with ITGβ3 using the Co-IP assay. BHK-21 cells were infected with DENV-2 and treated with R18. The ITGβ3-specific antibody was used to capture the E protein in BHK-21 cell lysates.

homogenized through sonication and subsequently clarified by centrifugation (13,000 × g at 37°C) for 30 min. The precipitate was dissolved in urea and loaded onto a pre-equilibrated NTA purification column. The column was eluted with NTAU-0 buffer [NTAU-X: 50 mM PBS (pH

7.4), 0.15 M NaCl, 8 M urea, and X mM imidazole], then sequentially eluted with NTAU-20, NTAU-50, NTAU-100, NTAU-200, and NTAU-500 buffers. Appropriate fractions were collected and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and

Coomassie brilliant blue (Beyotime, Shanghai, China) staining.

2.5 Surface plasmon resonance (SPR) screening

The interactions of 14 dammarane-type triterpenoid saponins with E and RdRp were analyzed using the PlexArray HT A100 system (Plexera Bioscience, Woodinville, WA, USA) [25]. The compounds were dissolved in 10 mM dimethyl sulfoxide (DMSO) and immobilized on a 3D photo-crosslinking chip in a dark room. The immobilization level was set to 100 response units (RUs). After vacuum drying for 30 min, the photo-crosslinked reaction of the chip was performed at 365 nm for 15 min. The chip was then sequentially rinsed with N, N-dimethylformamide, ethanol, and ultrapure water for 15 min. Recombinant ED III and RdRp proteins were diluted to 2000, 1000, 500, 250, and 125 nM with PBS in succession. R18 binding to ED III and RdRp was performed at various concentrations at a flow rate of 2 μ L/s for 300 s. Finally, the equilibrium dissociation constant (K_D) was calculated using BIAevaluation software (Cytiva, Marlborough, MA, USA) and the binding affinity curve was generated using OriginPro 8.5 software (OriginLab, Northampton, MA, USA).

2.6 Cellular thermal shift assay (CETSA)

The cells were lysed with RIPA lysis buffer supplemented with protease inhibitors and freeze-thawed three times in liquid nitrogen. After centrifugation, the cell lysates were incubated with R18 or DMSO as a control for 1 h at room temperature (RT). The treated cell lysates were subsequently divided equally into 8 PCR tubes and heated under various temperatures (40–75°C) for 5 min. After centrifugation, the supernatants were denatured with 1 $\times$ loading buffer for 5 min at 95°C and subjected to western blotting.

2.7 Drug affinity responsive target stability (DARTS)

The cells were harvested and lysed using M-PER lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA). Following quantification using a BCA protein assay kit (Thermo Fisher Scientific), the lysates were incubated with R18 or DMSO for 1 h at RT, then digested with different concentrations of pronase (Roche, Basel, Switzerland) for 15 min at RT. Next, the protein lysate was denatured at 95°C for 5 min after the addition of loading buffer and analyzed by western blotting.

2.8 Co-immunoprecipitation (Co-IP) assay

The cells were lysed using IP lysis buffer containing protease inhibitors, then centrifuged at 12,000 $\times$ g for 15 min at 4 °C. After quantification, the antibody against ITG β 3 was added to cell lysates and 30 μ L of Dynabeads Protein G (Thermo Fisher Scientific) was used to precipitate protein together with ITG β 3 overnight. After washing twice with IP lysis buffer, the beads were boiled in

2 $\times$ loading buffer for 5 min at 95 °C. The beads mixture was placed on DynaMag™ magnets (Thermo Fisher Scientific) to separate the beads and loading buffer containing proteins. The loading buffer was transferred into new tubes and analyzed for western blotting.

2.9 Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Recombinant E and RdRp proteins were incubated with R18 at 4 °C overnight. After denaturing at 95 °C with loading buffer, protein samples were subjected to 10% SDS-PAGE and stained with Coomassie brilliant blue. The gel bands containing E and RdRp proteins were cut into pieces, dehydrated, and reconstituted in sequencing-grade trypsin (Promega, Madison, WI, USA) for protein digestion. The tryptic peptides were subsequently extracted and lyophilized *via* vacuum centrifugation, then resuspended in water with 0.1% formic acid. LC-MS/MS analysis was operated on a nano-HPLC chromatography system (NanoElute; Bruker Corporation, Billerica, MA, USA) coupled with a hybrid trapped ion mobility spectrometry quadrupole time-of-flight mass spectrometer (TIMS-TOF Pro2; Bruker Corporation). The peptides were loaded onto an analytical column (75 μ m i.d. $\times$ 25 cm) and separated with a 60 min gradient (2%–22% solvent B [ACN with 0.1% formic acid] for 45 min, 22%–37% B for 5 min, 37%–80% B for 5 min, then 80% B for 5 min) at a flow rate of 400 nL/min. MS analysis was performed in the positive electrospray mode with the accumulation and ramp times at 100 ms. The data were obtained from 10 parallel accumulation-serial fragmentation MS/MS scans. The MS file was processed using Fragpipe (v19.0; Labsys Technologies, Seattle, WA, USA) for protein/peptide identification.

2.10 Molecular docking

The crystal structures of DENV-2 target proteins were downloaded from the RCSB Protein Data Bank (<https://www.rcsb.org/>) and the structure of R18 ligand was constructed using Chem3D 20.0 (PerkinElmer Informatics, Waltham, MA, USA). After hydrogenation and charge addition of target proteins in AutoDock 4.2.6 (Scripps Research, La Jolla, CA, USA), the grid center was created using the AutoGrid algorithm (Scripps Research). Finally, molecular docking was performed according to the AutoDock software operation guide (Scripps Research) and the docking results were displayed by Pymol software (Schrödinger, LLC, New York, NY, USA).

2.11 DENV-2 RNA-dependent RNA polymerase assay

The viral RNA-dependent RNA polymerase assay kit was purchased from ProFoldin (Hudson, MA, USA). The assay involves quantifying the RNA molecules produced by the RNA polymerase, which utilizes RNA as a template in the presence of ribonucleoside triphosphates (NTPs). Briefly, a premixed solution containing buffer, template,

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and recombinant RdRp was incubated with R18 for 10 min, followed by addition of the substrate NTP to initiate the enzymatic reaction. An equivalent volume of fluorescent dye was incorporated into the reaction mixture. The fluorescence intensity was detected at 535 nm upon excitation at 485 nm in 5 min.

2.12 Tryptophan fluorescence analysis

The purified RdRp protein was mixed with R18 (12.5, 25, 50, and 100 μ M) or an equivalent volume of DMSO, then placed in a shaker at 4 °C for slow incubation overnight. Aliquots of the protein mixture (100 μ L) were subsequently transferred to a black 96-well microplate in triplicate replicates. The fluorescence spectra were acquired using a Flash Spectrum multifunctional microplate reader (Shanpu, Shanghai, China) with the following parameters: detection height set at 1 mm; excitation wavelength at 280 nm; and emission wavelength scanning from 300–500 nm in 1-nm increments. The fluorescence intensity of samples was systematically recorded across the entire emission spectrum (300–500 nm) under standardized measurement conditions.

2.13 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT) assay

The cells seeded in 96-well plates were treated with different concentrations of R18 for 48 or 96 h. Then, 20 μ L of MTT solution (5 mg/mL) was added to each well and incubated for 4 h. Next, the supernatant was discarded and the formazan crystals were dissolved in 100 μ L of DMSO. Finally, the absorbance was measured using a microplate reader (Thermo Fisher Scientific) at 570 nm.

2.14 Time-of-drug-addition assay

R18 was administered at different time points of DENV infection to assess the stage of the DENV life cycle at which R18 acts. BHK-21 cells were treated prophylactically with R18 (0.78–25 μ M) 5 h prior to infection. After exposure to 200 DENV-2 plaque-forming units (PFUs)/mL for 1 h at 37°C, the cells were cultivated for 4 d in RPMI-1640 medium supplemented with 2% FBS. BHK-21 cells were inoculated with 200 DENV-2 PFUs/mL with or without R18 for 1 h at 4 °C for the binding assay. After twice-washing with PBS, the cells were incubated in RPMI-1640 medium containing 2% FBS at 37°C for 4 d. BHK-21 cells were inoculated with 200 DENV-2 PFUs/mL and incubated at 4°C for 1 h for the entry assay. The viral solution was aspirated, supplemented with RPMI-1640 medium containing 2% FBS and R18, and incubated for 1 h at 37°C. After removing the supernatant, the cells were further cultured with RPMI-1640 medium containing 2% FBS for 4 d. BHK-21 cells were infected with 200 DENV-2 PFUs/mL for 1 h at 37°C for the virus intracellular replication assay, followed by replacing the viral inocula with RPMI-1640 medium containing 2% FBS and R18 for 4 d.

2.15 CCK-8 assay

DENV-2-infected groups exhibited extensive CPEs following 96 h of cell culture. At this time point, 10 μ L of CCK-8 reagent (APExBio, Houston, TX, USA) was added to each well, followed by incubation at 37°C for 1 h. The absorbance at 450 nm was measured using a microplate reader (Thermo Fisher Scientific).

2.16 Plaque assay

BHK-21 cells seeded in 6-well plates were inoculated with DENV-2 and R18 was added for 48 h as described above. The cell supernatants were collected to infect new plated BHK-21 cells for 1 h at 37°C. Cells were washed twice using PBS, followed by the addition of RPMI-1640 medium containing 1.2% methylcellulose and 2% FBS. Following incubation for 7 d, 4% formaldehyde and 2% crystal violet were used for fixation and staining for 15 min, respectively. Finally, the plaques induced by progeny virus were photographed.

2.17 Quantitative real-time PCR (qRT-PCR)

Total RNA from cells and tissues was extracted with Trizol reagent (Takara, Kyoto, Japan), while serum viral RNA was extracted using the E.Z.N.A. Viral RNA kit® (Omega Bio-tek, Norcross, GA, USA), in accordance with the manufacturer's instructions. RNA concentration was measured using a DS-11+ spectrophotometer (DeNovix, Wilmington, DE, USA). For first-strand complementary DNA (cDNA) synthesis, 1000 ng of total RNA was reverse-transcribed into cDNA using the PrimeScript™ RT Master Mix (Takara) protocol. qRT-PCR was performed using a LightCycler® 96 real-time PCR instrument (Roche) with Bestar qPCR Master Mix® (DBI Bioscience, Shanghai, China). The probe and primer sequences used in this study were designed and synthesized by Thermo Fisher Scientific (Invitrogen™) as shown in Table S1.

2.18 Western blotting

Total protein of cells and tissues was lysed using RIPA lysis buffer containing a protease inhibitor. Protein samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). The membranes were then closed with 5% skim milk and sequentially incubated with the appropriate primary and secondary antibodies. The proteins were visualized using ECL reagent via the FluorChem E system™ (ProteinSimple, San Francisco, CA, USA).

2.19 Immunofluorescence analysis

After DENV-2 infection and addition of R18, cell fixation and permeabilization were performed using 4% paraformaldehyde (PFA) for 10 min and 0.1% Triton-100 for 5 min, respectively. Then, cells were blocked using 5% skim milk, followed by incubation with primary antibodies at 4°C overnight. After three rinses with PBS, the cells were incubated with Alexa Fluor 488-conjugated anti-rabbit IgG (Thermo Fisher Scientific) or Alexa

Fluor 555-conjugated anti-mouse IgG antibody (Thermo Fisher Scientific), then stained with DAPI for 5 min. The images were captured by confocal microscopy (LSM800; Carl Zeiss, Oberkochen, Germany).

2.20 Mice

ICR suckling mice (6 d old) were purchased from SiPeiFu Biotechnology Co., Ltd. (Beijing, China). AG129 mice (6–8 weeks old) were obtained from Professor Jianping Zuo, a co-author of this study. All mice were raised in the Biosafety Level II Laboratory Animal Center at Guangzhou University of Chinese Medicine (Guangzhou, China) and experiments were performed following approval by the Ethics Committee of Guangzhou University of Chinese Medicine (Permit numbers 20210518004 and 20230330003 for ICR suckling and AG129 mice, respectively).

2.21 Construction of a DENV infection murine model

ICR suckling and AG129 mice were randomly divided into 3 groups ($n = 8$ per group). ICR suckling mice were inoculated intracerebrally (400 DENV-2 PFUs/mL [20 μ L]) and intraperitoneally (4×10^5 DENV-2 PFUs/mL [50 μ L]) or the same volume of normal saline. R18 (3 mg/kg) or the same volume of normal saline was administered once every 2 d by intraperitoneal injection 1 h post-infection (hpi). The body weights, clinical scores, and suckling mouse mortality were recorded daily. The clinical scores were graded as previously described [26]. Finally, the mice were sacrificed 6 d post-infection (dpi) and the serum and organs, including brain, liver, kidneys, and spleen, were collected.

AG129 mice were injected intraperitoneally (4×10^5 DENV-2 PFUs/mL, 200 μ L) or the same volume of normal saline. At 1 hpi, R18 (3 mg/kg) or an equal amount of normal saline was given once every 2 d through intraperitoneal injection. The tails of mice were clipped to collect blood to assess viremia and the viral load in the blood. Finally, the liver and spleen tissues were isolated for histologic analysis.

2.22 Histopathology and immunohistochemistry

The tissues fixed with PFA were dehydrated in an ethanol series and embedded in paraffin and sectioned into 4- μ m sections. Finally, the dried tissue sections were stained with hematoxylin & eosin (H&E). For the immunohistochemistry assay, deparaffinized and hydrated tissue sections were treated with citric acid solution for antigen retrieval and hydrogen peroxide for endogenous peroxidase removal. After exogenous blocking with 5% bovine serum albumin, the tissues were incubated with the E protein antibody overnight and HRP-labeled polymer for 30 min. Finally, the sections were stained with 3, 3'-diaminobenzidine for 5 min and the positive expression and distribution of E proteins were observed under a digital pathology section scanner (KF-PRO-005; KFBIO, Yuyao, China).

2.23 Statistical analysis

Data are expressed as the mean $\pm$ standard deviation (SD) from three independent experiments. Data were analyzed using GraphPad Prism 8.0 (San Diego, CA, USA). Student's t-test was used for comparisons between two groups and one-way analysis of variance (ANOVA) was used for comparisons involving three or more groups. Kaplan-Meier analysis was used for determining the differences in mortality between groups. A $P < 0.05$ was considered statistically significant.

3. RESULTS

3.1 Identification of R18, which directly targets E and RdRp

Given the important roles of E and RdRp in viral entry and replication, inhibitors from *Panax notoginseng* targeting these two proteins were screened using SPR analysis (Figure 1B); compound 9 (R18), which simultaneously targets E and RdRp (Figure 1C), was identified. R18 was shown to exhibit strong binding affinity for the target proteins (ED III and RdRp) with equilibrium dissociation constants (K_D) of 0.84 μ M and 58.6 nM, respectively (Figures 1D, 2A).

3.2 R18 competitively binds E protein with ITG β 3

Two label-free experiments (CETSA and DARTS analysis) were performed to confirm the interaction between R18 and E protein. R18 protected against temperature-induced protein degradation and enhanced the thermal stability of E protein after DENV infection (Figure 1E). R18 decreased the susceptibility of E protein and prevented pronase from degrading the protein (Figure 1F, G).

LC-MS/MS analysis was performed to experimentally identify the amino acid sites responsible for R18 binding to ED III. The exact mass of R18 is 916.54 and the two major fragmentation peaks (F1 and F2) have exact masses of 789.48 and 347.10 under MS/MS conditions, resulting in residual masses of 127.08 and 569.44 (F3 and F4), respectively (Figure S2A, B). We speculated that ED III and RdRp may be covalently modified by the above five fragments. The peptide, VQYEGDGSPCK of ED III, displayed molecular weights of 1182.51 Da and 1309.59 Da in the absence and presence of R18, respectively, and this mass difference was consistent with the fragmentation peak (F3) of R18. In addition, the results of MS/MS suggested that the mass shifted from y2-y10, indicating the cysteine in the peptide (C333 in E protein) was covalently bound by R18 (Figure 1H, I). Furthermore, to ascertain whether additional non-covalent interactions contributed to the formation of the R18-ED III complex, molecular docking simulations were performed. R18 also interacted with amino acid residues surrounding the reactive C333. Specifically, R18 formed hydrogen bonds with glutamic acid 383 (E383) and glutamine 386 (Q386), as shown in Figure 1J. Taken together, these findings suggested that R18 was covalently bound to the C333 residue of E protein.

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The residue in E protein (Cys333) is situated in the region where E protein interacts with ITGβ3. Previous reports demonstrated that ITGβ3 mediates virus endocytosis in host cells by interacting with clathrin, which is closely related to the adsorption and entry process of the virus [27, 28]. We speculated that R18 might bind the E protein and prevent the interaction of the E protein with ITGβ3, thereby inhibiting viral entry into the cells. To test this hypothesis, the interaction between E protein and ITGβ3 was evaluated using the Co-IP assay. Antibody specific for ITGβ3 was able to capture E protein in DENV-infected cell lysates and this interaction appeared to be attenuated after administration of R18 (Figure 1K). These findings collectively implied that E protein interacts with ITGβ3, while R18 competitively binds to E protein to prevent the E protein- ITGβ3 interaction.

3.3 R18 allosterically regulates RdRp conformation to inhibit enzyme activity

Considering the polymerase function of RdRp, whether R18 binding to RdRp affects enzyme activity was determined. R18 remarkably decreased RdRp activity with an inhibition rate of 51.17% at 25 μM (Figure 2B). CETSA and DARTS assays were performed to verify the interaction between R18 and RdRp. The data showed that R18 increased the thermal stabilization and decreased the proteolytic susceptibility of NS5 (Figure 2C-E). Due to the presence of two structural domains (RdRp and MTase) in NS5 (Figure 2F), plasmids for these two proteins were constructed and CETSA and DARTS experiments were performed to confirm whether R18 selectively targets RdRp. R18 also protected RdRp from high temperature and pronase-induced degradation (Figure 2G-I) but had no apparent protective effect on MTase (Figure S3).

LC-MS/MS analysis showed that the mass of the peptide, GSGQVGTYGLNTFTNMEAQLIR, in RdRp was calculated to be 2357.15, which increased by 127.08 with the addition of R18. The mass shift from y12-y21 revealed that asparagine in this peptide (N339 in RdRp) was covalently modified by R18 (Figure 2J, K). Similarly, two other RdRp peptides, LAANAICSAVPSHWVPTSR and VWIQENPWMEDK, were identified in which cysteine (C509 in RdRp) and asparagine (N550 in RdRp) were modified by R18, respectively (Figure S4A-D). Next, molecular docking was performed to identify other non-covalent interactions with R18. R18 formed hydrogen bonds with tryptophan 267 (W267), aspartic acid 268 (D268), threonine 340 (T340), and aspartic acid 392 (D392), as shown in Figure 2L. Taken together, these data demonstrated that R18 covalently binds RdRp protein at the N339, C509, and N550 residues.

Non-nucleotide inhibitors (NNIs) that target RdRp mostly impede RdRp enzyme activity by altering the conformation [29]. We hypothesized that R18 might bind RdRp and regulate the conformation. Thus, we first performed a tryptophan fluorescence analysis using purified RdRp protein and found a dose-dependent decrease in the fluorescence intensity of tryptophan upon administration

of R18, suggesting that R18 induced RdRp conformational changes (Figure 2M). Overall, the above data suggested that R18 directly targets RdRp, altering the conformational transition to inhibit enzyme activity and functioning as an NNI to prevent DENV replication.

3.4 R18 protects against DENV-2-induced cell death at multiple stages of viral infection

The toxicity of R18 in BHK-21 cells was evaluated *in vitro* using the MTT assay to determine the antiviral effect of R18. R18 at concentrations ≤ 25 μM did not affect the proliferation of BHK-21 cells (Figure S5A). The life cycle of DENV infection in host cells mainly involves virus adsorption, entry, biosynthesis, assembly, and release of virus. R18 was added at different time points of infection to preliminarily evaluate the antiviral effect of R18 using the CCK-8 assay. Figure 3A schematically illustrates this time-of-drug-addition protocol. Pre-administration of R18 for 5 h failed to reduce DENV-induced cell death (Figure S5B). BHK-21 cells were co-treated with DENV-2 and R18 for 1 h at 4 °C to allow virus adsorption without entry or infected with DENV for 1 h at 4 °C, then R18 at 37 °C was added for another 1 h to allow the adsorbed virus to enter the host cell. R18 was shown to reduce DENV-induced cell death (Figure 3B, C).

The antiviral effects of R18 at viral binding and entry stage were then determined. Plaques caused by progeny viruses significantly declined as the R18 concentration increased (Figure 3D). Moreover, DENV-infected cells showed typical CPEs, manifested by wrinkled cellular morphology, decreased intercellular connectivity leading to cell detachment, membrane degradation, and ultimately death, which was reversed by co-treatment with R18 (Figure 3E). E protein is essential in inducing the body to produce neutralizing antibodies against virus infection. NS1 protein has a major role in viral replication and can be a critical indicator for the early diagnosis of viral infections. R18 activity in inhibiting DENV *E* and *NS1* genes was then assessed using a qRT-PCR assay. R18 co-treatment reduced gene copy numbers of *E* and *NS1* in a concentration-dependent manner (Figure 3F). Similarly, synthesis of *E* and *NS1* protein was restrained after R18 treatment, as confirmed by western blot and immunofluorescence analyses (Figure 3G-I).

In addition, R18 significantly blocked the intracellular replication of DENV in cells treated with R18 for 1 h after virus inoculation (Figure 4A). Post-R18 treatment also notably decreased the CPEs and progeny virus-induced plaques, exhibiting a comparable efficacy to the positive control, ribavirin (RV), as shown in Figure 4B, C, and decreased the number of RNA copies and levels of *E* and *NS1* protein (Figure 4D-G). In addition, a large amount of double-stranded RNA (dsRNA), the intermediate of viral replication, is produced during the process of viral replication. Fluorescence of dsRNA was nominal after the R18 intervention (Figure 4H). Typically, viral genomic RNA is translated into protein within 1–5 h after virus infection, viral RNA is synthesized after 5 hpi,

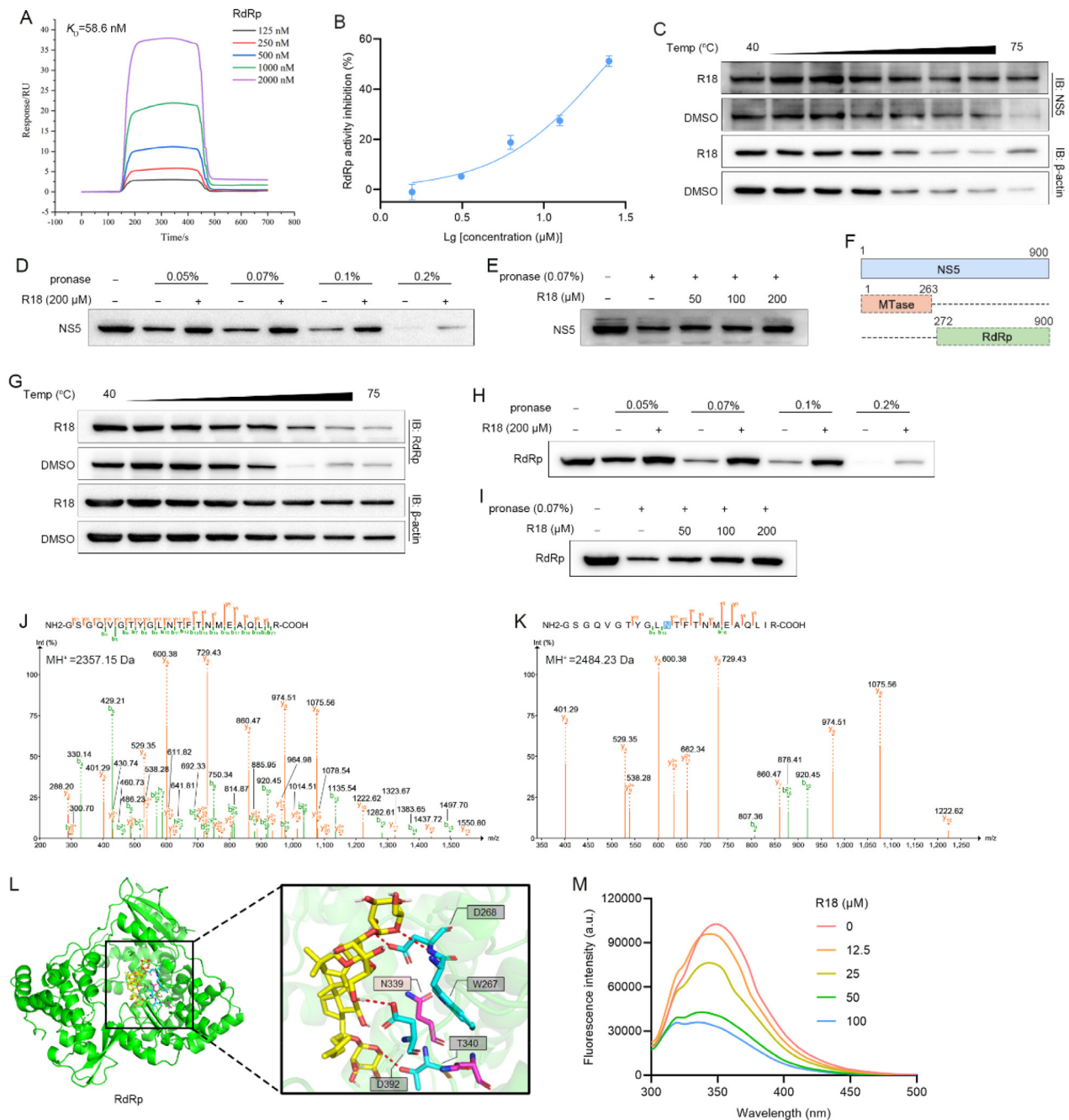


Figure 2 | R18 induces conformational changes of RdRp to inhibit enzyme activity.

(A) The binding affinity of R18 to RdRp detected by the SPR assay. (B) R18 inhibits the enzyme activity of RdRp. (C) R18 enhances the thermal stability of NS5 protein. The DENV-2-infected BHK-21 cell lysates were incubated with R18 or DMSO followed by CETSA. (D, E) R18 decreases the susceptibility of NS5 protein to pronase. The DENV-2-infected BHK-21 cell lysates were incubated with R18 followed by pronase digestion. (F) Schematic representation of the two structural domains of NS5 (MTase and RdRp). (G) R18 increases the thermal stabilization of RdRp. Flag-tagged RdRp plasmid was transfected into BHK-21 cells and cell lysates were collected for CETSA. (H, I) R18 increases the resistance of RdRp against proteases. Flag-tagged RdRp plasmid was transfected into BHK-21 cells and cell lysates treated with R18 or DMSO were incubated with pronase. (J, K) R18 covalently modifies RdRp at residue N339. Recombinant RdRp was incubated with R18 for 12 h, followed by SDS-PAGE and LC-MS/MS analysis. (L) The interaction diagram of R18 and RdRp determined by molecular docking. (M) R18 reduces the tryptophan fluorescence intensity of RdRp.

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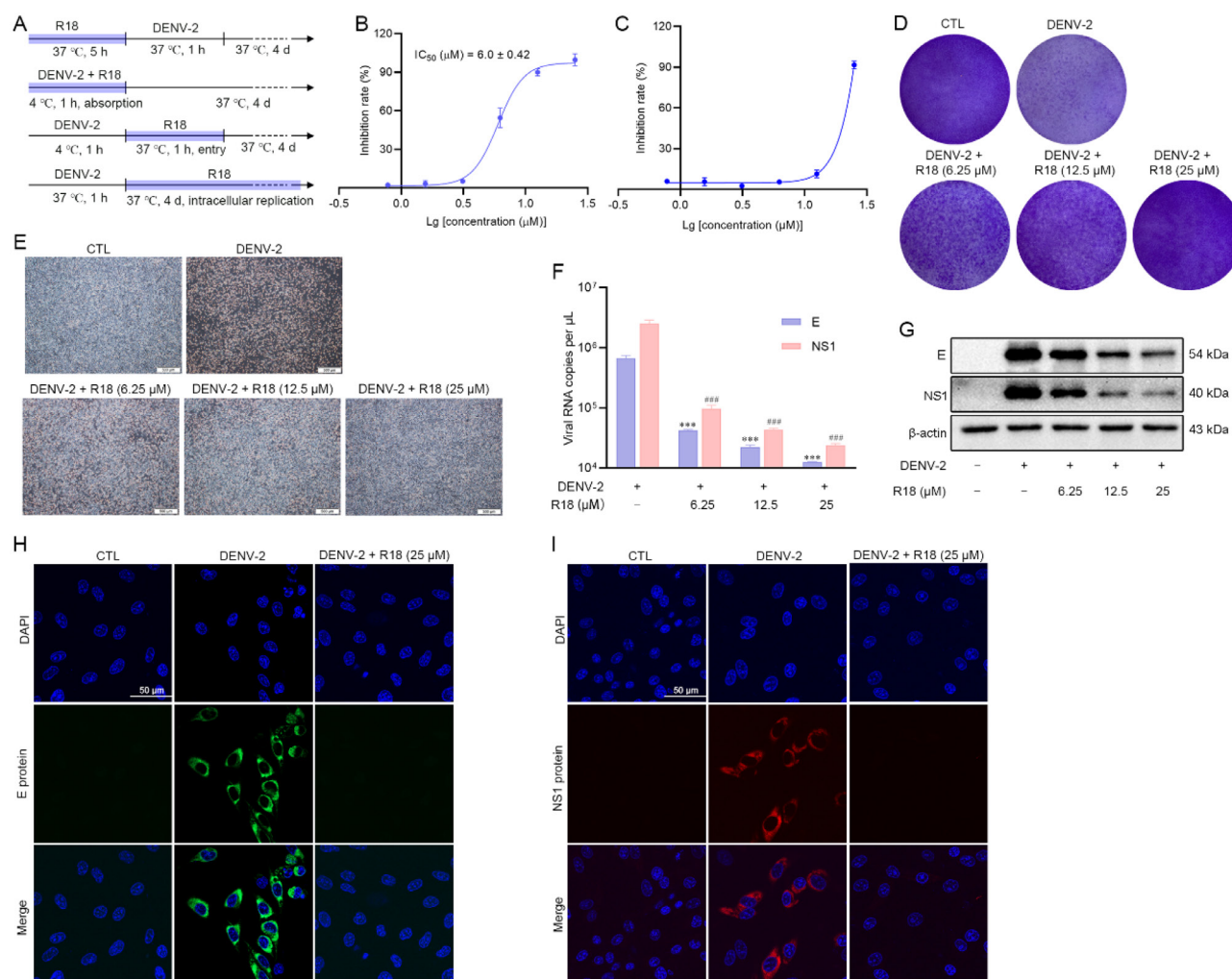


Figure 3 | R18 prevents DENV-2 binding and entry into BHK-21 cells.

(A) Schematic diagram of time-of-drug-addition assay. (B, C) Viral inhibition rates adsorption and entry stages following R18 administration. BHK-21 cells were infected with DENV-2 and treated with R18. Cell viability was detected using the CCK-8 assay. (D) R18 inhibits the release of progeny virus from DENV-2-infected cells. The plaques induced by progeny virus were visualized by crystal violet staining. (E) R18 decreases DENV-2-induced CPEs at adsorption and entry stages. (F) R18 reduces gene copy numbers of viral E and NS1. RNA expression was detected by qRT-PCR. (G-I) R18 decreases the levels of viral E and NS1 protein. Protein expression was determined by western blot and immunofluorescence assays. Data are expressed as the mean $\pm$ SD. *** $P < 0.001$ vs. the RNA levels of E in the DENV group; ### $P < 0.001$ vs. the RNA levels of NS1 in the DENV group.

and progeny viruses are assembled and released after 12 hpi. A time-of-drug-addition experiment was performed to determine at which stage of intracellular replication R18 potency is exerted. Inhibition of viral RNA synthesis was most pronounced when the drug was administered 0–8 hpi, suggesting that R18 mainly acts at the early stage of intracellular replication (Figure 4I). These findings demonstrated that R18 exhibits antiviral effects at multiple stages of viral infection.

3.5 R18 exhibits antiviral efficacy in different DENV serotypes and cell lines

The DENV-3 H87 strain and monkey-derived cell line (Vero) as well as human-derived cells lines (A549 and

Huh7) were used to determine if the antiviral effect of R18 could be generalized to other DENV serotypes and different cell lines. Co-administration or post-infection administration of R18 led to a reduction in the viral copy number of DENV-3, suggesting that R18 prevents adsorption and entry of DENV-3 and inhibits intracellular replication of DENV-3 (Figure 5A, B). The results of the MTT assay showed that R18 does not affect cell proliferation at concentrations $\leq 25 \mu M$ (Figure 5C). The results from the qRT-PCR assay also confirmed that DENV effectively propagated on these cells, whereas R18 significantly reduced the viral RNA copy number (Figure 5C-E). Together, these results indicate that R18 might display a broad spectrum of antiviral effects against DENV.

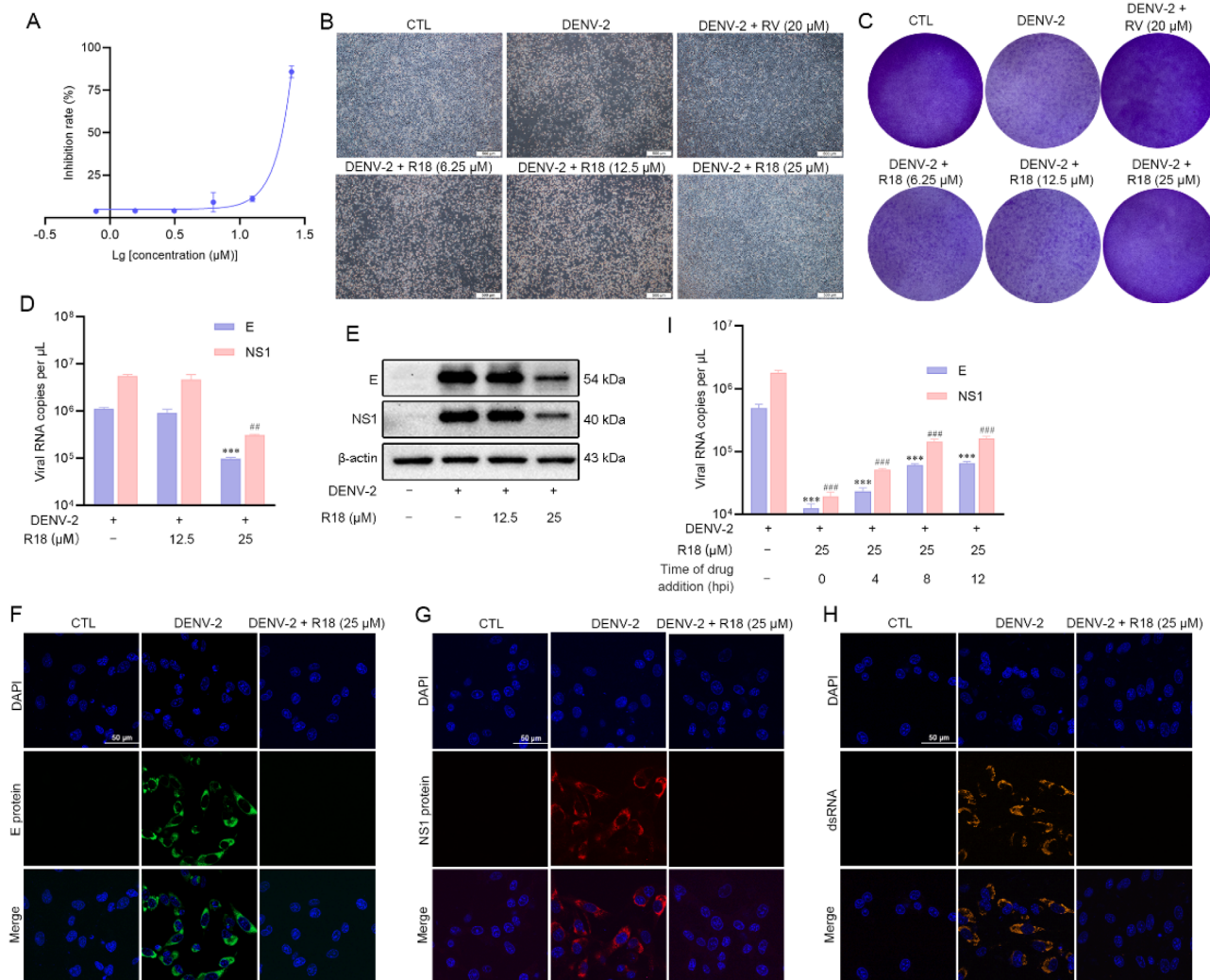


Figure 4 | R18 blocks the intracellular replication of DENV-2.

(A) Viral inhibition rate of R18 when administered after viral entry. The cell viability was detected by the CCK-8 assay. (B) R18 eliminates DENV-2-induced CPEs at the viral intracellular replication stage. (C) R18 inhibits the synthesis of progeny virus in DENV-2-infected cells. (D) R18 suppresses copy numbers of viral E and NS1. RNA expression was detected using the qRT-PCR assay. (E-G) R18 downregulates the expression of viral E and NS1 protein. The protein levels were detected by western blot and immunofluorescence analysis. (H) R18 inhibits the synthesis and distribution of dsRNA. (I) R18 is mainly involved in the early stages of intracellular replication. RV, ribavirin. Data are expressed as the mean $\pm$ SD. *** P < 0.001 vs. the RNA levels of E in the DENV group; ## P < 0.01, ### P < 0.001 vs. the RNA levels of NS1 in the DENV group.

3.6 R18 ameliorates the symptoms in DENV-infected ICR suckling and AG129 mice

A DENV infection murine model was established by simultaneous intracranial and intraperitoneal injection of DENV-2 in 7-day-old ICR suckling mice. R18 was administered intraperitoneally once every 2 days (Figure 6A). The results indicated that the infected mice began to exhibit severe clinical symptoms from 5 dpi, including weight loss, a bowed back, convulsions, limb weakness or paralysis, and ultimately death. Intervention with R18 significantly slowed the weight loss and the increase in clinical ratings, and prolonged the survival time in ICR

suckling mice (Figure 6B-D). The levels of viral E expression in the serum, brain, liver, spleen, and kidneys of infected mice were determined by qRT-PCR. R18 significantly reduced the viral copy number in serum and brain (Figure 6E, F) and also inhibited viral copy number in the liver, spleen, and kidneys (Figure 6G-I). The histopathologic results showed that DENV-2 infection caused severe damage to pyramidal neurons in the dentate gyrus (DG) and hippocampus from CA1-CA3. Inflammatory cell infiltration was noted in brain tissues with a large number of lymphocytes aggregating around blood vessels to form a perivascular cuff, which

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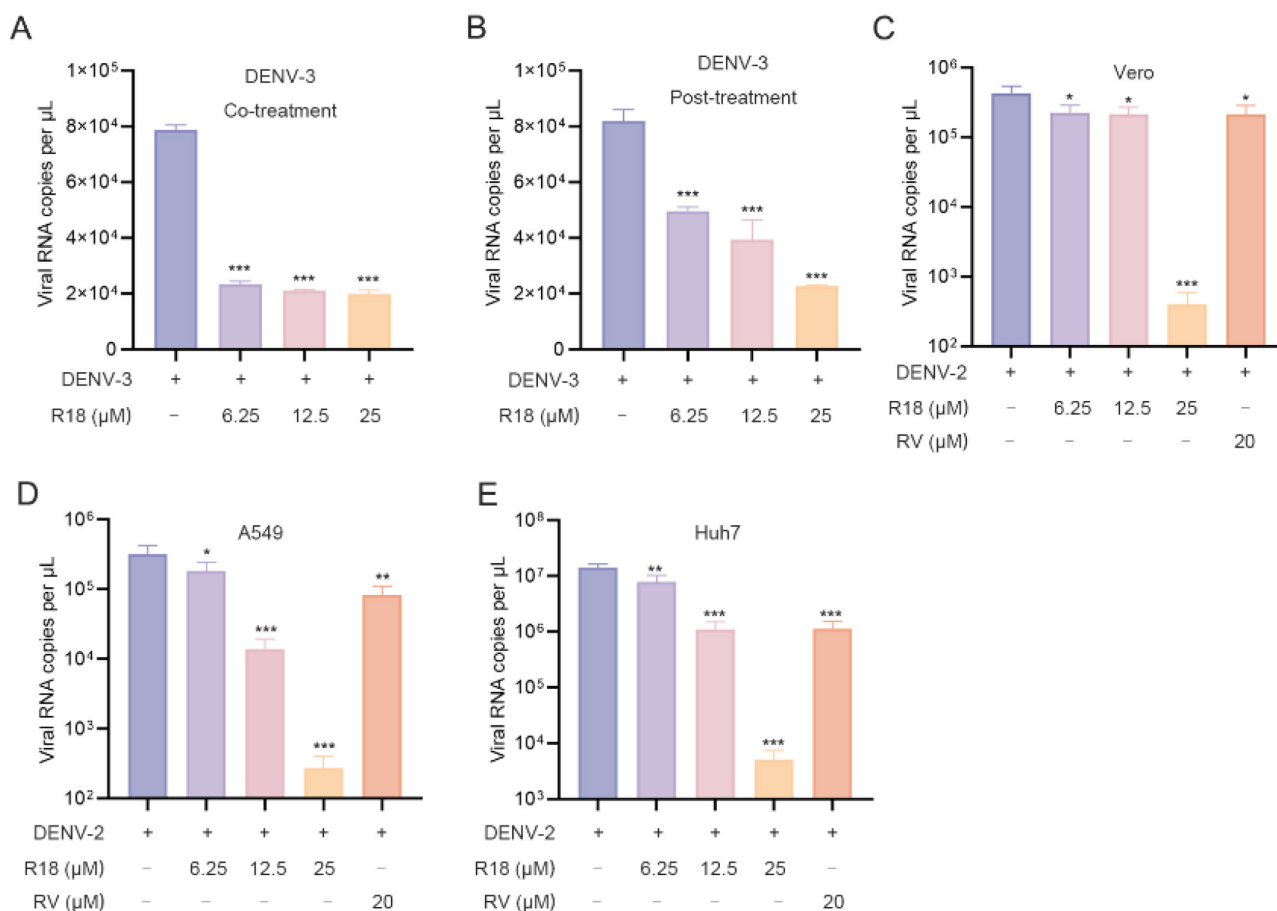


Figure 5 | R18 exhibits antiviral effects on different DENV serotypes and cell lines.

(A, B) R18 suppresses DENV-3 adsorption entry and intracellular replication. BHK-21 cells were infected with DENV-3 and treated with R18, followed by the qRT-PCR assay. (C-E) R18 reduced the viral copy number in DENV-2-infected Vero, A549, and Huh7 cells. Data are expressed as the mean $\pm$ SD. * P < 0.05, ** P < 0.01, *** P < 0.001 vs. the DENV-2 group.

were alleviated by R18 treatment (Figure 6J). Moreover, the results of immunoblot and immunohistochemistry analyses revealed that E protein was abundantly distributed in brain tissue, whereas R18 treatment reduced E protein expression (Figure 6K, L).

The antiviral efficacy of R18 was evaluated using virus-susceptible AG129 mice, which are defective in IFN- α/β and IFN- γ receptors (Figure 7A). DENV infection caused significant weight loss in AG129 mice as in ICR suckling mice and resulted in mortality starting on 10 dpi, whereas R18 treatment markedly improved the weight loss and survival (Figure 7B, C). Serum was collected from infected mice by tail clipping at 3, 5, and 7 dpi to assess the viral load in the blood. R18 significantly reduced the viral copy number at all time points, suggesting that viremia induced by DENV was attenuated (Figure 7D-F). The histopathologic results revealed that the viral infection caused swelling and hypertrophy of the liver cells, whereas R18 markedly ameliorated the pathologic alterations (Figure 7G). Spleen

pathology manifested primarily in the following three ways: structural disruption (blurring of the white/red pulp boundary); increased extramedullary hematopoiesis; and enhanced infiltration of megakaryocytes (evidenced by increased megakaryocytes, granulocytes, and nucleated erythrocytes, white arrow). Notably, R18 administration ameliorated these pathologic alterations (Figure 7H). Collectively, these results demonstrated that R18 reduced virus replication in ICR suckling and AG129 mice, thereby alleviating the symptoms induced by DENV.

4. DISCUSSION

DENV is classified into four serotypes (DENV-1–4) based on antigenic differences. Short-term infections with the same serotype are associated with mild symptoms, whereas infections with different serotypes may trigger ADE of infection, potentially resulting in the development of dengue hemorrhagic fever or dengue shock

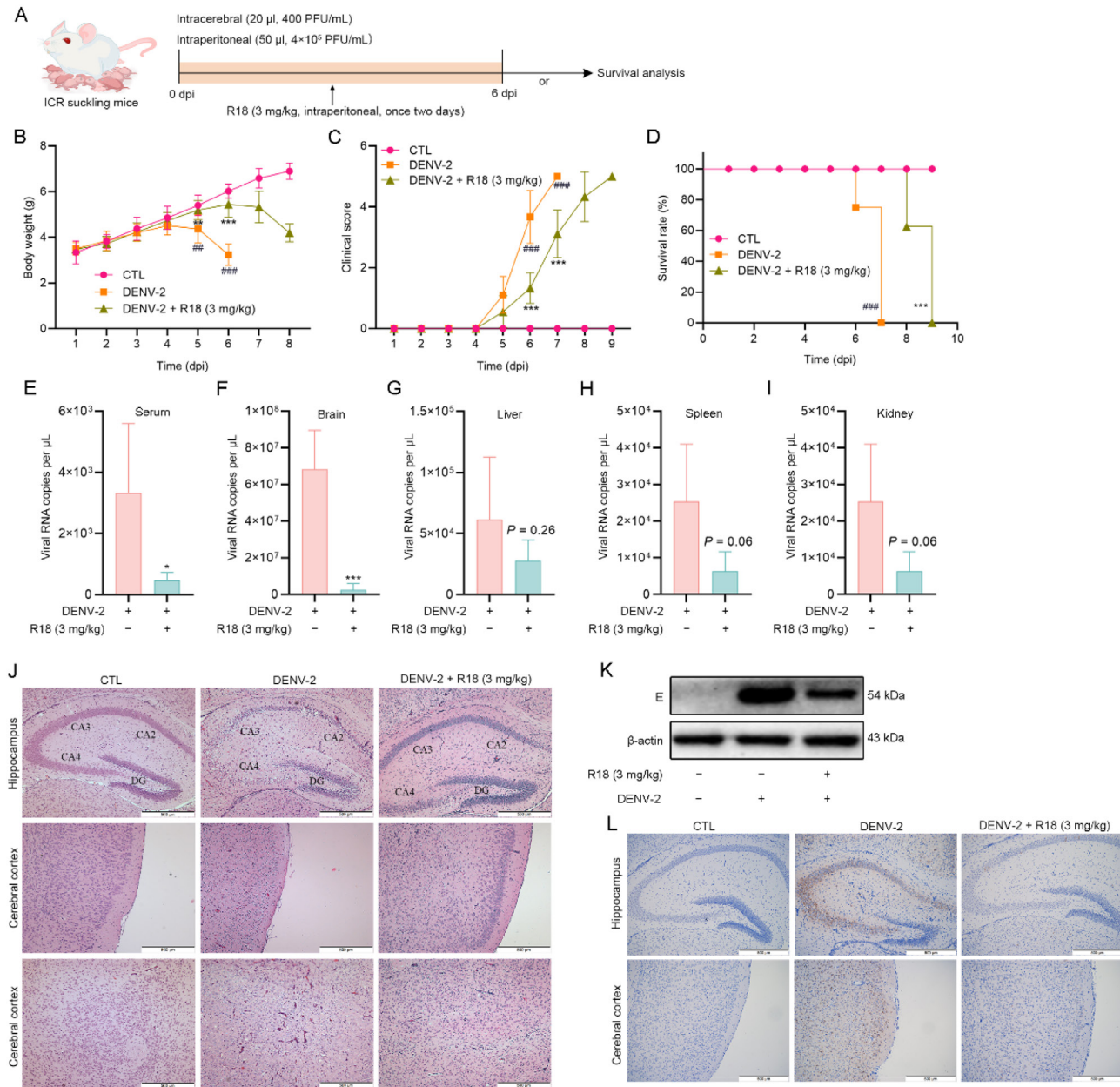


Figure 6 | R18 improves the symptoms of DENV-2-infected ICR suckling mice.

(A) Schematic representation of DENV-2 infection and R18 administration in ICR suckling mice. (B, C) R18 attenuates the weight loss and clinical ratings of ICR suckling mice. After infection, the body weights and clinical scores of mice were recorded daily. (D) R18 prolongs survival time of ICR suckling mice. (E, F) R18 significantly reduces viral load in serum and brain of ICR suckling mice. (G-I) R18 did not significantly reduce the viral load in liver, spleen, and kidneys of ICR suckling mice. Viral RNA expression was determined by qRT-PCR assay. (J) R18 improves the pathologic changes of brain in DENV-2-infected suckling mice. Histopathologic changes in the hippocampus and cerebral cortex were evaluated by H&E staining. (K, L) R18 reduces viral E protein level in brain tissue. The protein expression was detected by immunoblot and immunohistochemistry analysis. Data are expressed as the mean $\pm$ SD. $^{##}P < 0.01$, $^{###}P < 0.001$ vs. the control group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the DENV-2 group.

syndrome [30]. However, there is still a lack of effective antivirals to combat DENV infection. The only two approved dengue vaccines (Dengvaxia [CYD-TDV] and QDenga [TAK-003]), have considerably lower protection

in patients < 9 years of age and have a risk of ADE [31, 32].

Because numerous DAAs have achieved positive sustained virologic responses in clinical settings [33, 34],

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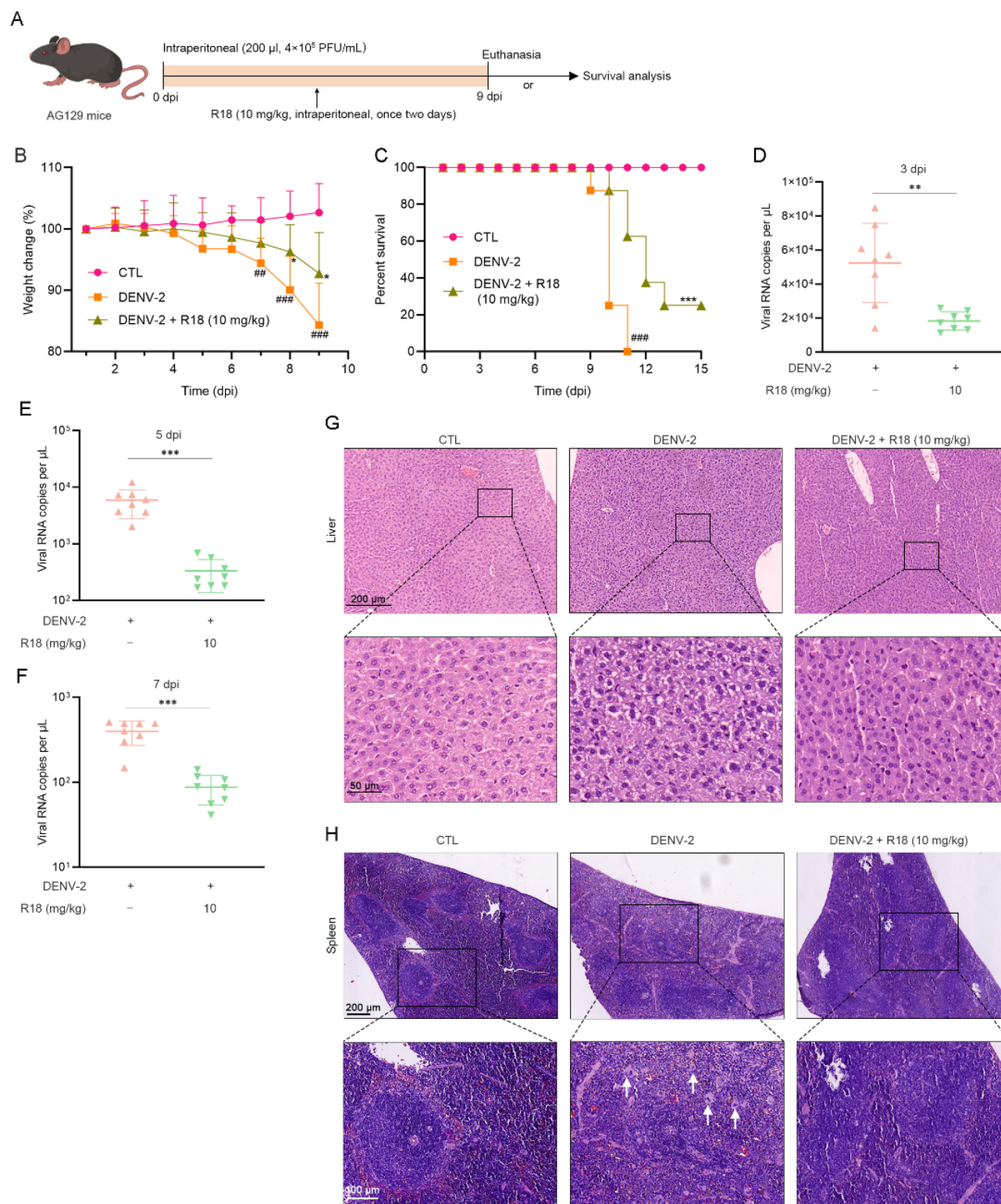


Figure 7 | R18 has a therapeutic effect on DENV-2-induced AG129 mice.

(A) The flowchart of DENV-2-infected AG129 mice and administration of R18. (B) Body weight of DENV-2-infected AG129 mice. (C) R18 improves AG129 mouse survival. The survival rates were measured using the Kaplan-Meier method. (D-F) R18 reduces the viral load in the blood at 3, 5, and 7 dpi. The virus RNA was extracted from mouse serum and subjected to qRT-PCR. (G, H) R18 ameliorates pathologic changes in the liver and spleen. The liver and spleen tissues were collected for H&E staining. Data are expressed as the mean $\pm$ SD. ## $P < 0.01$, ### $P < 0.001$ vs. the control group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. the DENV-2 group.

DAAAs are now being studied more closely in the search for antiviral medications. DAAAs primarily target proteins that have central roles in the viral life cycle, including the E protein, which mediates binding of the virus to cell receptors and allows the virus to enter host cells, and the RdRp domain of the NS5 protein, the core component of the viral replicase system [35, 36]. VIS513, a recombinant monoclonal antibody targeting ED III and neutralizing all four serotypes of DENV, is currently undergoing phase II clinical trials [37, 38]. Several RdRp antiviral inhibitors have been approved for treating SARS-CoV-2 infection and have shown significant clinical efficacy, including azvudine, molnupiravir, and deuremidevir hydrobromide [39]. However, there are no studies focusing on discovering dual-target inhibitors that target E and RdRp against DENV. In this study small molecules from Chinese medicine that specifically target ED III or RdRp were screened using the SPR assay and R18 was shown to have strong affinity for ED III and RdRp protein, indicating the potential of R18 as a novel DAA by simultaneously targeting E/RdRp. Considering that E and RdRp are mainly involved in virus adsorption for entry and replication processes, respectively, an *in vitro* time-of-drug-addition assay was performed and the data confirmed that R18 inhibits the proliferation of DENV at viral adsorption and entry, as well as intracellular replication stages, which partly supports R18 targeted E/RdRp. However, the primary focus of the current study was on the direct antiviral effects of R18 as a DAA against DENV. In future corollary studies, we plan to synthesize biotin-tagged R18 compound and use pull down coupled with LC-MS/MS experiments to identify potential host target proteins. This approach will enable us to explore whether R18 enhances antiviral activity by modulating host immune responses, offering valuable mechanistic insights into the R18 mode of action.

DENV, an RNA virus, has poor replication fidelity compared to DNA viruses, and is prone to viral mutations and development of resistance to existing antiviral drugs [40]. DAAAs that directly target viral proteins are more susceptible to resistance than HDAs, which act on host factors. To overcome this shortcoming, combination therapy with multiple drugs that act at different stages of the viral cycle appears to be an effective strategy. For example, the combination of favipiravir and oseltamivir prevents drug resistance caused by mutations of influenza viruses and shortens the clinical recovery time in patients with severe influenza compared to oseltamivir alone [41]. However, combining multiple drugs may produce more severe side effects than using a single drug due to possible drug-drug interactions that can affect the pharmacokinetic properties of the drugs [42]. These concerns have given rise to increasing attention regarding dual-target inhibitors, which avoid drug-drug interactions compared to drug combinations and have a higher barrier to resistance compared to single-target inhibitors. A previous study showed that emetine potently inhibits ZIKV infection

through the following two mechanisms: inhibition of ZIKV NS5 polymerase activity; and disruption of lysosomal function [20]. However, the exact amino acid binding sites of emetine on the NS5 protein remains elusive, which raises ambiguity regarding the interaction with conserved residues within NS5, and consequently, the durability of the resistance barrier. In the current study ED III and RdRp proteins were identified as targets of R18, indicating the potential of R18 as a dual-target inhibitor. Moreover, the RdRp amino acids (N339, C509, and N550) identified as the binding sites of R18 are all strictly conserved, implying the potential of R18 against the emergence of drug resistance.

NNIs mainly occupy the allosteric pocket of RdRp to regulate the conformation and further decrease enzyme activity. A high-throughput fragment library screen conducted by researchers identified RK-0404678 as a potent NNI of DENV RdRp. The co-crystal structure analysis comprehensively elucidated the unique binding mode to RdRp and an adaptation experiment confirmed that RK-0404678 exhibits a high genetic barrier to the development of resistance [43]. However, RK-0404678 has not been validated in mice to have anti-DENV effects, which is a major challenge in the current research involving this compound. The antiviral potency of R18 was comprehensively characterized in the current study based on integrated *in vitro* and *in vivo* investigations, demonstrating robust suppression of viral replication across preclinical models. Moreover, R18 was shown for the first time to not only bind to target sites (C509 and N550) to change the conformation of RdRp, but also interact with N339, which is located in the catalytic region and responsible for recognizing and binding RNA replication substrate NTP [44]. This finding implies that R18 possesses the properties of NNIs to change the RdRp conformation and has the characteristics of nucleoside inhibitors to directly affect enzyme activity. In addition, the amino acids (N339 and N550) have high homology among multiple flavivirus genomes [45], indicating that R18 might exert a broad antiviral effect on the other flaviviruses by binding to RdRp.

Research has demonstrated that the R18 target site (C333) in ED III is situated in the region where E protein interacts with ITGβ3 [27, 28]. ITGβ3 is a cell surface receptor for many viruses. Previous studies have shown that ITGβ3 is a receptor for DENV-2-infected endothelial cells and blocking the interaction sites between DENV-2 and ITGβ3 with peptides prevents DENV-2 entry into cells [27]. The results of the current study revealed that R18 attenuates the interaction of E protein and ITGβ3 by acting at the C333 residue, which might be caused by steric hindrance on the interaction domain, thereby inhibiting the adsorption and entry of DENV to prevent the host cells from being infected. Taken together, these results demonstrated that residues targeted by R18 (specifically, C333 in the E protein and N339, C509, and N550 in the RdRp) represent novel druggable sites for the design of antiviral lead compounds. Moreover, the conserved

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structures and functions of DENV E protein and RdRp across flaviviruses suggest that R18 may demonstrate broad-spectrum antiviral activity against related viruses, including ZIKV, West Nile virus, and yellow fever virus.

DENV-2 is currently recognized as the most widespread serotype worldwide and is frequently linked to more severe secondary infections. DENV-3 also represents a major serotype with high infectivity [46]. Due to limitations in experimental resources and time, the current study mainly focused on evaluating the efficacy of R18 against DENV-2 and DENV-3. We plan to expand our experiments in future corollary studies to include all four DENV serotypes and comprehensively assess the broad-spectrum antiviral activity of R18. Because rodents are not sensitive to DENV, establishing animal models suitable for DENV infection has been a challenge. We first used immunologically intact ICR suckling mice to construct a dengue infection model. Previous studies involving ICR suckling mice focused on detecting pathologic changes in the brain with less attention paid to the overall systemic pathology [47, 48]. In the present study high DENV copy numbers were detected in the serum, as well as the liver, spleen and kidney tissues. Notably, the DENV copy numbers were decreased by R18 treatment. However, no significant pathologic damage was demonstrated in other organs except the brain. This finding could be interpreted as a higher viral load in the brain resulting in death before the virus caused substantial damage to the organs. AG129 mice, which are more susceptible to the virus, were also used to continue evaluating the *in vivo* antiviral effect of R18. The mice were shown to exhibit significant viremia, which was significantly alleviated by R18 intervention. Given that high viremia is a risk factor for severe dengue progression, quantifying viral RNA copies in serum as a key metric was a specific focus rather than other hematologic parameters, such as white blood cell or platelet counts. Liver and spleen injury were significant clinical manifestations associated with DENV infection, in agreement with findings from prior published research [49-51]. However, a low level of viral RNA copies in the liver and spleen was measured, which may be attributed to the natural infection progression characterized by a peak followed by a gradual decline in viral load after 3 dpi. Nonetheless, this reduced viral load does not preclude DENV from triggering a robust immune response that may account for the liver and spleen damage that occurred. Moreover, the liver pathology may also be correlated with liver transaminase enzymes and alkaline phosphatase. We plan to incorporate broader hematologic assessments and liver biochemical markers to better characterize hepatic responses to DENV infection and correlate the findings with histopathologic outcomes.

While our research has highlighted the therapeutic promise of R18 against DENV infection in murine models, it is crucial to acknowledge the notable disparities in DENV pathogenesis between these animals and humans. Notably, there are substantial differences

in the composition and function of the immune system across various species. For example, the AG129 mice used in this study lack a fully functional immune response, which may limit the ability to accurately replicate the intricate immunopathologic mechanisms observed in humans. In addition, humans infected with DENV typically exhibit symptoms, such as fever, bleeding, thrombocytopenia, and shock syndrome. In contrast, mice may display weight loss, reduced activity, and increased viral loads, but often fail to fully replicate these complex clinical manifestations. Vascular leakage, a key feature of severe dengue, poses challenges in accurate simulation within mouse models. Moreover, determining the therapeutic window of R18 is crucial for the potential human translation. In future studies we plan to systematically evaluate the pharmacokinetic and pharmacodynamic properties of R18 to assess the potential for clinical application. Further validation in models that more closely resemble humans, such as non-human primates, will be crucial. This analysis will enable us to predict human exposure and establish a dose-translation strategy based on the FDA-recommended body surface area normalization method, thereby determining a clinically relevant human dose.

5. CONCLUSION

We were the first to identify a dual-target compound (R18) that simultaneously targets DENV E protein and NS5 RdRp. To the best of our knowledge, this is the first dual-target inhibitor of E/RdRp against DENV infection. Further mechanistic studies showed that R18 inhibited the interaction between ED III and ITGβ3 and altered RdRp conformation to decrease enzyme activity. Consequently, DENV infection was significantly suppressed *in vivo* and *in vitro* at the virus adsorption and entry stages, as well as the replication stage. The findings of this study suggest the potential of R18 as a dual-target inhibitor against DENV infection and also demonstrate that searching for novel agents from triterpenoid saponins may be a new advance into the development of antiviral drugs.

ABBREVIATIONS

ADE, antibody-dependent enhancement; CETSA, cellular thermal shift assay; Co-IP, co-immunoprecipitation; CPE, cytopathic effects; DAAs, direct-acting antivirals; DARTS, drug affinity responsive target stability; DENV, dengue virus; DMSO, dimethyl sulfoxide; dsRNA, double-stranded RNA; E, envelope; ED III, envelope protein domain III; FBS, fetal bovine serum; H&E, hematoxylin & eosin; HDAs, host-directed antivirals; HIV, human immunodeficiency virus; HPLC, high-performance liquid chromatography; ITGβ3, integrin beta3; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MTase, methyltransferase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide; NGC, DENV-2 New Guinea C strain; NNIs, non-nucleotide inhibitors; PFA, paraformaldehyde; qRT-PCR, quantitative real-time PCR; R18, vira-ginsenoside R18;

RdRp, RNA-dependent RNA polymerase; RV, ribavirin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SPR, surface plasmon resonance; ZIKV, Zika virus

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

ICR suckling and AG129 mice were housed in the Biosafety Level II Laboratory Animal Center at Guangzhou University of Chinese Medicine (Guangzhou, China). All experiments were conducted in accordance with protocols approved by the Ethics Committee of Guangzhou University of Chinese Medicine (Permit numbers 20210518004 and 20230330003 for ICR suckling and AG129 mice, respectively).

AUTHOR CONTRIBUTIONS

Y.O., L.Y., J.L., Y.Z. and X.H. conceived and supervised the study; X.H., Y.Z., L.Z. and J.Y. performed the experiments, analyzed the data and wrote the manuscript; B.C., Z.G., G.P., Z.L., W.Z. and J.Z. participated in conducting experiments, provided intellectual expertise. All authors have read and approved the article.

ACKNOWLEDGEMENTS

The work was supported by the National Natural Science Foundation of China (81730110).

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

SUPPLEMENTARY MATERIAL

Supplementary Material can be downloaded from https://amm-journal.org/wp-content/uploads/2025/10/amm20240094_Suppl.pdf.

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