**Virologica Sinica**

**Supplementary Data**

***In vitro* and *in vivo* efficacy of** **Molnupiravir against Zika virus infections**

Zhuang Wang a, b, 1, Shaokang Yang b, c, 1, Qingsong Dai b, 1, Xiaojia Guo b,, Yuexiang Li b, Wei Li b, Xiaotong Yang b, e, Jingjing Yang b, d, Xintong Yan b, d, Huimin Tao b, e, Chongda Luo b, d, Song Li b, d, Xingjuan Chen a,\*,

Ruiyuan Cao b, \*, Wu Zhong b, \*

a Institute of Medical Research, Northwestern Polytechnical University, Xi’an, 710072, China;

b National Engineering Research Center for the Emergency Drug, Beijing Institute of Pharmacology and Toxicology, Beijing, 100850, China;

c College of Chemical and Pharmaceutical Engineering, Hebei University of Science and Technology, Shijiazhuang, 050018, China.

d Song Li’s Academician Workstation of Hainan University (School of Pharmaceutical Sciences), Yazhou Bay, Sanya, 572000, China.

e School of Medicine, Tsinghua University, Beijing, 100084, China

\* Corresponding authors:

Email addresses: xjchen@nwpu.edu.cn (X. Chen); 21cc@163.com (R. Cao); zhongwu@bmi.ac.cn (W. Zhong)

ORCID: 0000-0002-7380-8399 (X. Chen); 0000-0002-5459-4495 (R. Cao); 0000-0002-0536-620X (W. Zhong)

1 Zhuang Wang, Shaokang Yang, and Qingsong Dai contributed equally to this work.

**Materials and Methods**

**1. Cell, Viruses and Reagents**

The Vero cell line was purchased from the American Type Culture Collection. BHK and Huh7 cells were obtained from the National Infrastructure of Cell Line Resources (China). All cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 ℃, 5% CO2. The BHK21-ZIKV Replicon cell line kindly provided by Prof. Han-Qing Ye (Key Laboratory of Special Pathogens and Biosafety, Wuhan Institute of Virology, Chinese Academy of Science, Wuhan, China) was cultured in DMEM with 10% fetal bovine serum, 1% penicillin/streptomycin, and 0.8 μg/mL puromycin.

Flaviviruses including ZIKV (SMGC-1 strain), DENV (serotype 2, New Guinea C strain), YFV (17-D strain), and JEV (SA-14 strain) were stored in our laboratory. Virus titers were measured using the plaque-forming unit assay (PFU) or 50% tissue culture infective dose infectivity assay (TCID50).

Molnupiravir (EIDD-2801, TargetMol, cat #T8309), EIDD-1931 (TargetMol, cat #T8498), NITD008 (7-Deaza-2′-C-acetylene-adenosine, MCE, cat #HY-12957), and 2′-CMA (2′-C-methyladenosine, TargetMol, cat #T16325) were dissolved in dimethyl sulfoxide (DMSO) as 100 mmol/L stocks and stored at -20 ℃ for further study.

**2.*****In vitro* antiviral assay**

BHK and Huh7 cell lines were incubated in 96-well plates with threefold serially diluted compounds and ZIKV virions (5.62×104 TCID50 of viral stock was diluted to a 100 TCID50 working solution) until cytopathic effect (CPE) protection was observed. Cell viability was measured using the CellTiter-Glo Luminescent Cell Viability Kit (Promega, cat #G7572) according to the manufacturer’s instructions. And the 50% cytotoxic concentration (CC50) value was determined using a similar protocol, except for the addition of virus. The 50% inhibitory concentration (IC50) and CC50 values were calculated using Origin 9.0 software.

BHK cells were seeded in a 12-well plate, cultured overnight, and then inoculated with gradient-diluted EIDD-1931 in the presence of viruses [multiplicity of infection (MOI) = 0.05]. After incubation for 72 h, the supernatant was collected to detect infectious virus particles, and the cells were used to extract viral RNA and viral proteins.

**3. qRT-PCR**

Total cellular RNA or total blood RNA was extracted using TRIzol reagent and quantified by qRT-PCR according to the manufacturer’s instructions of the One Step PrimeScript RT-PCR kit (TaKaRa Bio, cat #RR064A). Viral RNA copies were determined from the cycle threshold value of each sample with reference to the known copy number standard curve. The specific primers and probes used for ZIKV were as follows:

ZIKV forward primer, 5′-GGTCAGCGTCCTCTCTAATAAACG-3′; Reverse primer, 5′-GCACCCTAGTGTCCACTTTTTCC-3′; Probe, FAM-AGCCATGACCGACACCACACCGT.

**4. Immunofluorescence assay**

BHK cells seeded in 96-well plates were inoculated with virus inoculum at an MOI of 0.05 PFU/cell. Diluted compounds or DMSO were added and co-incubated for 48h. After fixing with 4% paraformaldehyde, the cells were treated with 0.1% Triton X-100 to permeabilize the cell membrane and blocked with 5% bovine serum albumin (BSA) for 1h at room temperature. The primary antibody for mouse anti-flavivirus group antigen (1:500, Merck Millipore, cat #MAB10216) and the corresponding secondary antibody (1:500, Invitrogen, cat #A32727) were diluted and incubated sequentially with the cells. Cells were then washed, stained with 1 µmol/L Hoechst 33342 fluorescent stain (Thermo Fisher Scientific, cat #H21492), and photographed using a Leica DMi8 microscope.

**5. Plaque assay**

The supernatant harvested from the cultured infected cells was tenfold serially diluted in 2% DMEM. The diluted virus was added to a monolayer of Vero cells in a 12-well plate. After 2 h of incubation, the cells were washed twice and covered with 2 × DMEM containing 2% low-melting point agarose. After four days, the cells were fixed with 4% formaldehyde and stained with crystal violet. The number of plaques in each well was manually counted.

**6. Western blotting**

Total protein was quantified using a BCA protein assay kit (Applygen, cat #P1511) after collection using cell lysis buffer (Beyotime, cat #P0013). Equal amounts of proteins were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane. After blocking the washed membranes, the target proteins were detected by immunoblotting with anti-ZIKV NS5 antibody (GeneTex, cat #GTX133329) or anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Abcam, cat #ab8245) and the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody. Protein bands were imaged using a Fluorchem imaging system (ProteinSimple).

**7. Time-of-drug-addition assay**

BHK cells were seeded in 12-well plates at a density of 2.5 × 105 cells/well and cultured overnight. At 0 h, cells were inoculated with ZIKV (MOI = 0.05) and incubated for 2 h, EIDD-1931 (50 µmol/L) or the positive control drug NITD008 (5 µmol/L) was added at four time points corresponding to different phases of the viral life cycle. Stage Ⅰ represents cell pretreatment, stage Ⅱ represents virus adsorption and entry, stage Ⅲ represents the whole life cycle of virus invasion cells, and stage Ⅳ represents the replication stage of virus. After treatment, the cells in each group were washed three times with DMEM containing 2% fetal bovine serum. Total RNA was extracted from cells 24 h post-infection and subjected to qRT-PCR. The percentage infection rate was calculated as “viral RNA copies of the drug treated group / viral RNA copies of the control group”.

**8. ZIKV replicon inhibition assay**

Confluent BHK–ZIKV replicon cells (Li et al., 2018) were co-cultured with the gradient-diluted compounds for 48 h at 37 ℃. The cells were washed with PBS and measured using the Dual-Luciferase Reporter Assay System kit. The compound concentration required to inhibit 50% of the Rluc activity (IC50) was estimated using the Origin 9.0 software. The CC50 value was determined using the CellTiter-Glo Luminescent Cell Viability Kit and calculated using Origin 9.0 software. The inhibitor 2′-CMA was used as a positive control.

**9. *In vivo* antiviral efficacy**

All animals used in this study were specific pathogen-free, and all the experiments and operations were reviewed and approved by the Institutional Animal Care and Use Committee of Beijing Institute of Pharmacology and Toxicology. One-day-old ICR mice were used to evaluate the anti-ZIKV effects of EIDD-2801/EIDD-1931 *in vivo*. Each mouse was injected intraperitoneally (i.p.) with 25 µL of a viral stock containing 1.3 × 104 PFU of viral particles, followed by administration of various concentrations of the compounds or vehicle. The mice in each group were i.p. treated once daily for nine consecutive days. The survival rate and body weight of infected mice were recorded for 21 consecutive days.

For blood viral RNA level and tissue damage score, three groups of suckling mice, treated with 100 mg/kg EIDD-2801, 50 mg/kg EIDD-1931 or saline, were euthanized at day four post infection. The viral RNA in the blood from ZIKV-infected mice was measured using the qRT-PCR assay. The brain or liver tissue was stained with hematoxylin and eosin, and the histological scores were made by estimating tissue damage levels by a pathologist blinded to the study (0 = absent; 1 = light; 2 = moderate; 3 = severe).

**10. Statistical analyses**

Statistical analyses were performed using GraphPad Prism 7 software. Statistical differences were determined using unpaired two-tailed Student’s *t*-test or one-way analysis of variance. Survival curve was determined using the log-rank test. For each test, *P* < 0.05 indicated a statistically significant difference. \* *P* < 0.1, \*\* *P* < 0.01, \*\*\* *P* < 0.001