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Letter

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

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Dear Editor,

Zika virus (ZIKV) is a mosquito-borne, positive-stranded RNA virus first identified in 1947 in monkeys and later identified in humans in 1952 (Faye et al., 2014). It is among the "TORCH" group of microorganisms and causes outbreaks in several countries and regions since 2007 (Voordouw et al., 2019). Transmission of ZIKV has been reported in 87 countries/territories worldwide (WHO, 2022), including the Pacific Islands (Wikan and Smith, 2016) and Colombia, with more than 65,000 reported cases (Pacheco et al., 2020). ZIKV infection in humans generally results in a self-limiting illness, such as rash or fever (Lessler et al., 2016). It also induces testicular and spermatic damage, resulting in reduced fertility (Barzon et al., 2016; Nicastri et al., 2016; Nie et al., 2021). However, ZIKV infection was found to be associated with severe neurological diseases, such as Guillain-Barré syndrome, microcephaly, and congenital joint-limb contractures, and raised major public health concerns over the last decade. In Brazil, an estimated 5%–10% of babies born to mothers infected with ZIKV during pregnancy showed features of congenital Zika syndrome including microcephaly and/or neurodevelopmental delay (Cugola et al., 2016). Currently, there are no clinically approved vaccines or antiviral drugs for the treatment of ZIKV infection, which drawn urgently needs for the discovery and mechanism research of new anti-ZIKV drugs.

EIDD-2801 (Molnupiravir or MK-4482) is an oral prodrug of antiviral ribonucleoside analog EIDD-1931 (β-d-N4-hydroxycytidine, NHC), which targets viral RNA-dependent RNA polymerase (RdRp), resulting in viral genome mutations and chain termination (Fig. 1A). EIDD-1931 was originally developed for the treatment of influenza, while its prodrug EIDD-2801 significantly improved the oral bioavailability in a non-human primate model and has a broad-spectrum antiviral activity against several highly pathogenic RNA viruses *in vitro* and *in vivo*. EIDD-2801 exhibited favorable *in vivo* antiviral efficacy in a ferret influenza infection model (Toots et al., 2019). Wang et al. reported that EIDD-1931 significantly inhibited Crimean-Congo hemorrhagic fever virus (CCHFV) infection in Vero E6 cells (EC_{50} = 5.19 ± 1.43 μmol/L, SI = 46.72). However, neither EIDD-1903 nor EIDD-2801 showed any protective effect in CCHFV-infected mice (Wang et al., 2022a). Another recent study found that EIDD-1931 was effective against Ebola virus challenge with 92%–100% protection in a BALB/c mouse model (Bluemling et al., 2022). Moreover, EIDD-2081 treatment inhibited viral replication in the lungs of hamsters infected with SARS-CoV-2 variants of concern and, in particular, in the respiratory tract of hamsters infected with the coronavirus Omicron variant (Rosenke et al., 2022). Currently, Molnupiravir is approved for the oral treatment of SARS-CoV-2 infections in the United States, United Kingdom, and China. It is important to systematically evaluate the *in vitro* and *in vivo* antiviral efficacy against other viruses with pandemic potential including ZIKV.

Firstly, we tested the antiviral activities of EIDD-2801 and EIDD-1931 by performing cytopathic effect (CPE) protection assay in baby hamster kidney (BHK) cells using a variety of flavivirus strains (Yang et al., 2022), including ZIKV, dengue virus (DENV), yellow fever virus (YFV), and Japanese encephalitis virus (JEV). As showed in Table 1, EIDD-2801 and EIDD-1931 exhibited broad-spectrum anti-flavivirus effects in BHK cells under non-cytotoxic conditions, and EIDD-1931 showed better efficacy against ZIKV, DENV, and YFV at lower concentrations.

Next, we further evaluated the anti-ZIKV activities of EIDD-2801 and EIDD-1931 in different cell lines (Fig. 1B and C). BHK and Huh7 cell lines were incubated with gradient-diluted compounds and ZIKV virions (5.62×10^4 $TCID_{50}$ of viral stock was diluted to a 100 $TCID_{50}$ working solution) until cytopathic effect protection was observed. The results showed that the half-maximal inhibitory concentration (IC_{50}) values of

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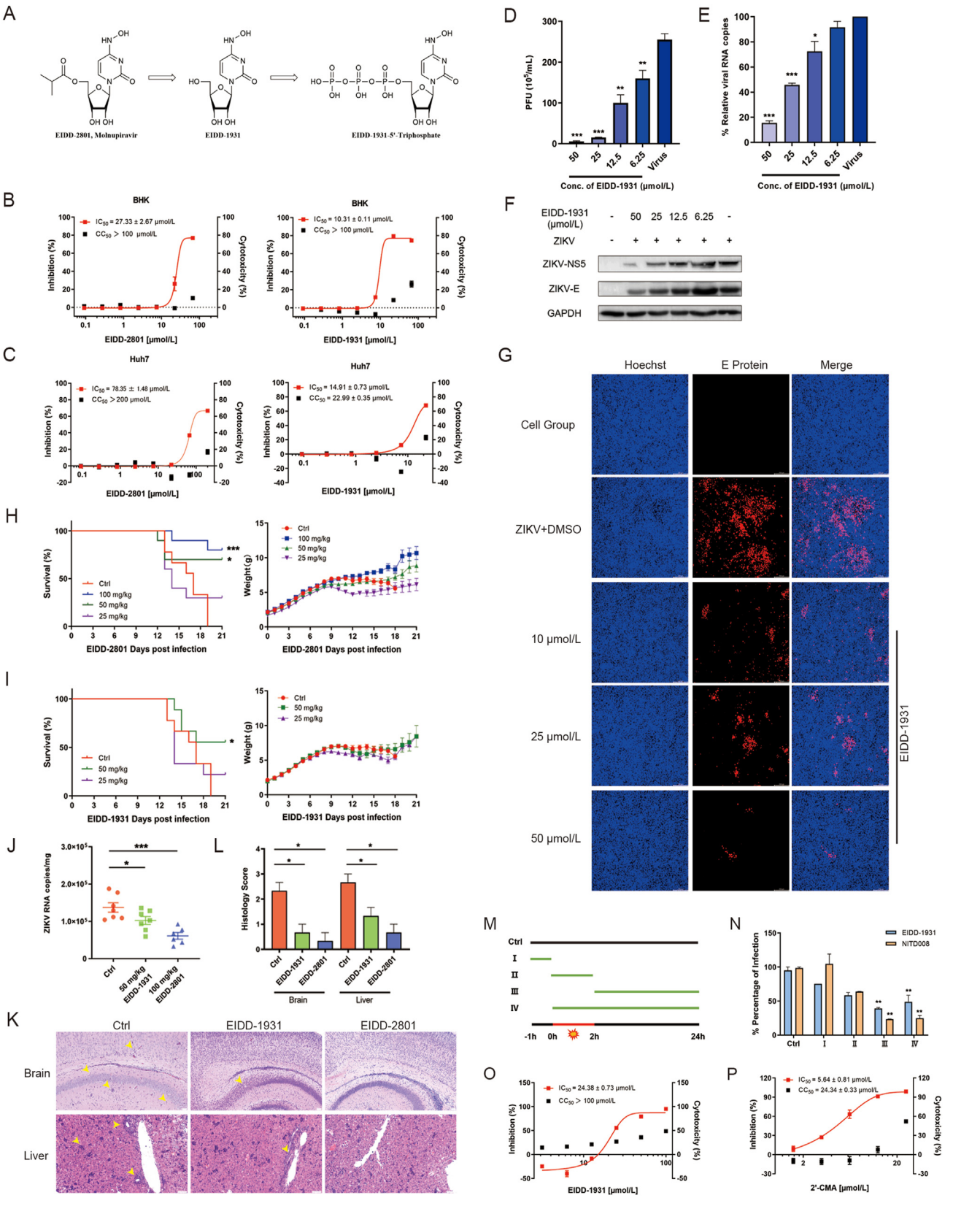

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Fig. 1. Molnupiravir inhibits ZIKV *in vitro* and *in vivo*. **A** *In vivo* metabolism of EIDD-2801. **B, C** The 50% inhibitory concentration (IC_{50}) and 50% cytotoxic concentration (CC_{50}) of EIDD-2801 or EIDD-1931 were evaluated in BHK (**B**) and Huh7 (**C**) cell lines, respectively. BHK and Huh7 cell lines were co-cultured with gradient-diluted compounds and 100 $TCID_{50}$ ZIKV virions until cytopathic effect (CPE) protection was observed. The left and right y-axes represent the mean % CPE inhibition and cytotoxicity of the drug, respectively. Data were obtained with three independent tests. **D–F** EIDD-1931 inhibited production of progeny virus particles, viral RNA and protein in a dose-dependent manner. BHK cells were inoculated with ZIKV (MOI = 0.05) in the presence of the gradient-diluted EIDD-1931. After 72 hours, the supernatant was collected to detect infectious virus particles (**D**) by viral plaque forming unit (PFU) assay, and the cells were used to extract and determine the viral RNA (**E**) and ZIKV structural and non-structural (E and NS5) protein level (**F**) by qRT-PCR and Western blotting respectively. **G** BHK cells were inoculated with virus inoculum at an MOI of 0.05 PFU/cell. Diluted compounds or DMSO were added and co-incubated for 48h. Cells were fixed for immunofluorescence assay and stained with Hoechst (blue) and anti-flavivirus envelope protein (red). Scale bar = 200 μm. **H, I** One-day-old ICR suckling mice were each challenged with 1.2×10^4 PFU ZIKV-SMGC-1 and injected intraperitoneally with indicated doses of EIDD-2801 (**H**), EIDD-1931 (**I**) or saline (control group) for nine consecutive days. The daily survival rate and changes of body weight of the mice were monitored for 21 days. Survival data were analyzed with a log-rank test. * $P < 0.1$, *** $P < 0.001$. **J** The viral RNA in blood from control, EIDD-2801 (100 mg/kg) or EIDD-1931 (50 mg/kg) treated mice was determined by qRT-PCR assay at day four post infection. Data were presented as means ± standard error of the mean (SEM). Student's unpaired *t*-test was performed for statistical analysis. * $P < 0.1$, *** $P < 0.001$. **K, L** Hematoxylin and eosin staining of sectioned brain and liver collected at day four post infection. Typical results (**K**) of tissue damage were presented, arrows indicated tissue damage. (Scale bar = 50 μm). Histological scores (**L**) were made by estimating tissue damage levels by a pathologist blinded to the study (0 = absent; 1 = light; 2 = moderate; 3 = severe). **M** Schematic representation of time-of-drug-addition assay. BHK cells were seeded and cultured overnight. The virus (MOI = 0.05) were added at 0–2 h (red line), and the addition of drugs at different time periods (I–IV) was done to inhibit different stages of the viral life cycle. The control group (ctrl) was treated with no drug during the whole process. The inhibitor NITD008 was used as the positive control. **N** Levels of intracellular viral RNA were quantified by qRT-PCR at 24 hours post infection. The percentage infection rate was calculated as 'viral RNA copies of the drug treated group/viral RNA copies of the control group'. The data collected from three independent experiments were represented as mean ± SEM. Statistical significance was analyzed using one-way analysis of variance. ** $P < 0.01$. **O, P** EIDD-1931 inhibition of ZIKV replicon. Confluent BHK–ZIKV replicon cells were co-cultured with the gradient-diluted compounds at 37 °C and luciferase activities were measured at 48 h.p.i. The inhibitor 2′-CMA was the positive control drug. The inhibition curve was fitted by GraphPad Prism 7 software.

Table 1
Broad-spectrum anti-flavivirus potential of EIDD-2801 and EIDD-1931.

Flavivirus	EIDD-2801		EIDD-1931	
	IC_{50} (μmol/L)	CC_{50} (μmol/L)	IC_{50} (μmol/L)	CC_{50} (μmol/L)
ZIKV	27.33 ± 2.67	> 200	10.31 ± 0.11	> 100
DENV	> 66.67	> 200	14.65 ± 0.16	> 100
YFV	> 200	> 200	24.14 ± 17.49	> 100
JEV	> 200	> 200	>66.67	> 100

IC_{50} and CC_{50} values were determined in three independent experiments. The data were presented as the mean ± standard deviation (SD).
Abbreviations: Zika virus (ZIKV), dengue virus (DENV), yellow fever virus (YFV), Japanese encephalitis virus (JEV), half-maximal inhibitory concentration (IC_{50}), and 50% cytotoxic concentration (CC_{50}).

EIDD-2801 in BHK and Huh7 cells were 27.33 ± 2.67 μmol/L and 78.35 ± 1.48 μmol/L, respectively. For EIDD-1931, the IC_{50} values were 10.31 ± 0.11 μmol/L and 14.91 ± 0.73 μmol/L, respectively. Besides, both compounds showed acceptable *in vitro* safety profiles.

We then used EIDD-1931, the *in vivo* active form of EIDD-2801, to further explore the antiviral potential (Toots et al., 2019). First, we performed qRT-PCR and viral plaque forming unit (PFU) assays. BHK cells were inoculated with ZIKV (MOI = 0.05) in the presence of the gradient-diluted EIDD-1931. After 72 hours, the supernatant was collected to detect infectious virus particles by PFU assay, and the cells were used to extract and determine the viral RNA and protein level by qRT-PCR and Western blotting, respectively. As expected, EIDD-1931 significantly inhibited the production of progeny viral particles (Fig. 1D) and the replication of viral RNA (Fig. 1E). As shown in Fig. 1F, 50 and 25 μmol/L EIDD-1931 treatment reduced viral structural (E protein) and non-structural protein (NS5 protein) expression, which was consistent with the results of the viral RNA and PFU assays. Furthermore, an immunofluorescence assay was performed to visualize the inhibition of ZIKV by diluted EIDD-1931 in BHK cells. The result showed that EIDD-1931 inhibited viral protein production in a dose-dependent manner (Fig. 1G). The collective findings indicate the favorable *in vitro* anti-ZIKV activities of EIDD-1931.

A fatal one-day-old ICR suckling mouse ZIKV challenge model (Wang et al., 2022b) was used to evaluate the anti-ZIKV efficacy of EIDD-2801 and EIDD-1931 *in vivo*. The suckling mice were randomly grouped, followed by intraperitoneal (i.p.) injection with 1.2×10^4 PFU of ZIKV per mouse. The mice were then i.p. injected with 100, 50 or 25 mg/kg EIDD-2801, 50 or 25 mg/kg EIDD-1931, or saline (control group) daily for nine consecutive days (Li et al., 2022). Treatment with 100 mg/kg EIDD-2801 protected 80.0% of the mice from lethal challenge of ZIKV (Fig. 1H). EIDD-1931 also showed dose-dependent protection. An administration with 50 mg/kg EIDD-1931 protected 55.6% of mice from death. The body weight of mice in the EIDD-1931 and EIDD-2801 treated groups increased, while decreased body weight was observed in the saline-treated group (Fig. 1H and I). To further characterize the *in vivo* antiviral effects of EIDD-2801 and EIDD-1931, the viral RNA in blood from ZIKV-infected mice was measured using the qRT-PCR assay at day four post infection. As shown in Fig. 1J, ZIKV RNA levels were significantly reduced in the 100 mg/kg EIDD-2801 treated and 50 mg/kg EIDD-1931 treated groups. In addition, hematoxylin and eosin staining of brain or liver tissue collected at day four post infection showed an alleviated tissue damage in the drug treatment groups (Fig. 1K and L) (Wang et al., 2018). Taken together, the above results indicated that EIDD-2801 and EIDD-1931 possess promising *in vivo* antiviral activities.

We next conducted a time-of-drug-addition assay to explore the stage of action of Molnupiravir. As shown in the schematic diagram (Fig. 1M), with ZIKV added at 0–2 h, EIDD-1931 was added at different time points corresponding to the different stages of the viral life cycle (Yan et al., 2022). In Fig. 1N, both EIDD-1931 and NITD008 (a positive control drug acting on the viral RNA replication stage) reduced viral RNA at stage III/IV, suggesting that EIDD-1931 might act on the post-entry step of ZIKV. The inhibitory effect of EIDD-1931 was also evaluated using a BHK cell line possessing a ZIKV replicon (BHK-ZIKV replicon cells) (Li et al., 2018). As expected, EIDD-1931 inhibited the replication of ZIKV replicon (IC_{50} = 24.38 ± 0.73 μmol/L, SI > 4.10) as did the positive control drug 2′-CMA (IC_{50} = 5.64 ± 0.81 μmol/L, SI = 4.32) (Fig. 1O and P). These results suggest that the anti-ZIKV activities of EIDD-1931 and EIDD-2801 involve the genome replication stage of ZIKV.

ZIKV is a highly focused flavivirus with global pandemic potential (Sargent et al., 2022). In this study, we evaluate the anti-ZIKV activity of Molnupiravir and expand its antiviral spectrum. The findings also demonstrate that Molnupiravir blocks ZIKV infection by inhibiting viral genome replication throughout the viral life cycle, with potent *in vitro* and *in vivo* efficacy. As a nucleoside analog, it probably acts by interfering with viral RdRp and induces genome mutation or chain termination. However, the specific mode of Molnupiravir needs to be further verified using the crystal structure of ZIKV RdRp. In this study, we provide experimental evidence that Molnupiravir is a promising antiviral drug candidate for flaviviruses including ZIKV. Considering that Molnupiravir is an approved novel antiviral drug that can be orally administered, it is of importance to conduct further clinical studies to evaluate its clinical efficacy against flavivirus infections.

Footnotes

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