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Effectiveness of favipiravir (T-705) against wild-type and oseltamivir-resistant influenza B virus in mice

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ABSTRACT

The emergence of resistant mutants to the wildly used neuraminidase inhibitors (NAIs) makes the development of novel drugs necessary. Favipiravir (T-705) is one of the RNA-dependent RNA polymerase (RdRp) inhibitors developed in recent years. To examine the efficacy of T-705 against influenza B virus infections in vivo, C57BL/6 mice infected with wild-type or oseltamivir-resistant influenza B/Memphis/20/96 viruses were treated with T-705. Starting 2 h post inoculation (hpi), T-705 was orally administered to mice BID at dosages of 50, 150, or 300 mg/kg/day for 5 days. Oseltamivir was used as control. Here, we showed that T-705 protected mice from lethal infection in a dose-dependent manner. T-705 administration also significantly reduced viral loads and suppressed pulmonary pathology. In addition, phenotypic assays demonstrated that no T-705-resistant viruses emerged after T-705 treatment. In conclusion, T-705 can be effective to protect mice from lethal infection with both wild-type and oseltamivir-resistant influenza B viruses.

1. Introduction

Influenza viruses are the causative pathogens of seasonal flu epidemics and occasional influenza pandemics (Medina and Garcia-Sastre, 2011; Molinari et al., 2007). WHO estimates that epidemics annually result in about 3–5 million cases of severe illness, and about 290,000 to 650,000 respiratory deaths worldwide. The current circulating seasonal influenza virus includes A(H1N1), A(H3N2) and type B. Previous report showed that influenza B virus-induced cases make up 29% of influenza-related deaths in an average season, and this proportion has measured up to 95% when influenza B was in high circulations (Matias et al., 2014). To treat influenza virus infections, there are two main types of drugs available. Owing to the appearance of widespread drug resistance and the lack of efficacy against influenza B viruses, M2 ion-channel blockers (amantadine and rimantadine) are no longer recommended to use (Bautista et al., 2010; Webster and Govorkova, 2014; Zaraket et al., 2010); thus, NAIs (oseltamivir, zanamivir, peramivir and laninamivir) become the major class of antiviral agents (Ling et al., 2010; Liu et al., 2014). However, the emergence of NAI-resistant mutants can reduce their efficacy (Boivin, 2013; Webster and Govorkova, 2014). The global analysis of NAI susceptibility of influenza viruses has been conducted since 2012 (Takashita et al., 2020). Frequency of viruses with reduced susceptibility to NAIs has remained low since then and low numbers of NAI-resistant B/viruses, with 43 in 5881 viruses tested, have been detected in the last year (Takashita et al., 2020). However, the emergence of NAIs resistant virus is still worthy of concern. Moreover, meta-analysis of published data displayed that influenza B viruses are less susceptible to NAIs compared with influenza A viruses (Burnham et al., 2013; Yen, 2016). Therefore, the study on novel antivirals to influenza B virus is essential.

Favipiravir, also known as T-705, targeting the RdRp of influenza viruses, inhibits influenza A, B and C viruses, including oseltamivir-resistant strains (Delang et al., 2018; Furuta et al., 2002, 2013; Manicassamy et al., 2014; Smee et al., 2009). T-705 has been tested to be efficacious against B/Brisbane/60/2008 in mice (Pascua et al., 2019) and oseltamivir-resistant influenza B viruses in vitro including B/Memphis/20/96(R152K) (Sleeman et al., 2010; Takashita et al., 2016), however, its efficacy against oseltamivir-resistant influenza B viruses has never been demonstrated in animal models. In this study, we evaluated the effect of T-705 on wild-type and oseltamivir-resistant influenza B viruses using a lethal mouse model. In addition, we assessed whether mutations inducing reduced susceptibility to T-705 occurred

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following drug treatment in vivo.

2. Materials and methods

2.1. Ethics statement

All mouse experiments were conducted after the approval of the Ethics Committee of the National Institute for Viral Disease Control and Prevention, China CDC (20190912031) in a biosafety level 2 facility.

2.2. Viruses, cells and compounds

The influenza viruses B/Memphis/20/96 and oseltamivir-resistant variant B/Memphis/20/96(R152K) (Sleeman et al., 2010) were from the US CDC and stored by the Chinese National Influenza Center. Virus stock was grown in Madin-Darby canine kidney (MDCK) cells for 3 days at 35 °C in serum-free Dulbecco's modified Eagle's medium (DMEM; Invitrogen, USA) supplemented with 2 μg/mL tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin. MDCK cells were cultured at 37 °C in DMEM supplemented with 10% fetal bovine serum (FBS; Invitrogen), 100 U/mL penicillin, 100 μg/mL streptomycin and 25 mM HEPES buffer (Invitrogen).

T-705, oseltamivir carboxylate and oseltamivir were purchased from MCE company. For in vitro experiments, T-705 was dissolved in DMSO as a 10 mM stock and was diluted with DMEM when used. For mouse experiments, T-705 was suspended in 0.4% carboxymethyl cellulose (CMC). Oseltamivir carboxylate and oseltamivir were prepared in sterile distilled water.

2.3. Treatment of mice

Specific-pathogen-free, female 8-week-old C57BL/6 mice were intranasally inoculated with 50 μl of two 50% mouse lethal doses (MLD_{50}) of viruses under isoflurane anaesthesia. Starting 2 hpi, mice were orally administered T-705 at dosages of 50, 150, or 300 mg/kg/day or oseltamivir at the dosage of 60 mg/kg/day BID (at 12 h intervals) for 5 days. T-705 was suspended in 0.4% CMC for animal studies, so infected, untreated control animals orally received 200 μl of 0.4% CMC twice daily for 5 days. Body weights and survival of infected mice were monitored for 18 days post inoculation (dpi) daily (n = 9/group). Mice that lost more than 30% of their initial weight were euthanized. The body weight change of mice was calculated and shown as a percentage of its initial weight.

2.4. Crystal digital PCR assay

At 3, 5, and 7 dpi, lungs, nasal turbinates and tracheas were harvested and homogenized in 1 mL of PBS containing penicillin and streptomycin (n = 3/day/group). After cellular debris was removed by centrifugation at 10,000 rpm for 1 min, supernatants were used to quantify the viral loads by digital PCR assay. Viral RNA was extracted from lung homogenates using the MagMAX™ CORE Nucleic Acid Purification kit (Applied Biosystems, USA). PCR reaction was performed using qScript™ XLT 1-Step RT-qPCR ToughMix (Quanta Biosciences, MD, USA). The crystal digital PCR workflow and data analyses were carried out using the Naica Geode and Naica Prism3 (Stilla Technologies, Villejuif, France) as previously described (Jovelet et al., 2017; Madic et al., 2016).

2.5. Lung cytokines and chemokines measurements

The supernatants of lung homogenates at 3, 5, and 7 dpi were tested for cytokines and chemokines. For IL-1β, IL-6, IFN-γ, macrophage inflammatory protein-1α (MIP-1α) and monocyte chemoattractant protein-1 (MCP-1), ELISA was conducted according to the manufacturer's instructions (Dakewe, China).

2.6. Cell viability assay to determine T-705 susceptibility of viruses recovered from lung

MDCK cells in 12-well plates were washed twice with PBS and then used to isolate influenza viruses from 400 μl of lung homogenates collected at 5 dpi. After 1 h of virus adsorption, supernatant was removed; cells were washed twice with PBS and cultured in fresh serum-free DMEM medium supplemented with 2 μg/mL TPCK-treated trypsin at 35 °C for 72 h. After 72 h, supernatant containing influenza virus was titrated in MDCK cells in 96-well plates. The 50% tissue culture infectious dose ($TCID_{50}$) was calculated with the Reed and Muench formula (Reed and Muench, 1938). Then, MDCK cells were seeded in 96-well plates (1.5×10^4 cells/well), and inoculated with recovered influenza viruses at the multiplicity of infection (MOI) of 0.01 $TCID_{50}$/cell. After 72 h incubation in the presence of T-705 (0.1–100 μM), cell viability was measured with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent (Promega, WI, USA) (Cao et al., 2014; Jones et al., 2017). GraphPad Prism 5.0 software was used to calculate 50% effective concentration (EC_{50}).

2.7. Neuraminidase inhibition assay

According to the manufacturer's instructions, the susceptibility of influenza B viruses to oseltamivir carboxylate was detected by the NA-Fluor™ Influenza Neuraminidase Assay Kit (Applied Biosystems, MA, USA). The fluorescence signal was measured with Envision (PerkinElmer, USA). EC_{50} was determined by GraphPad Prism 5.0 software.

2.8. Lung histopathology

For lung tissues, conventional paraffin embedding was performed with right upper leaf of lung of mice sacrificed on 3, 5, and 7 days (n = 3/group). The tissues were fixed in 10% paraformaldehyde. Then paraffin blocks were cut into sections which were then stained with hematoxylin and eosin (H&E). The extent and severity of lung damage was assessed in a blinded fashion.

2.9. Serology test for anti-hemagglutinin (HA) antibodies

At 21 dpi, serum samples were obtained from mice that survived influenza virus infection. Then mice sera were treated with receptor-destroying enzyme (Denka Seiken, Japan) at 37 °C for 16–18 h, heat-inactivated for 30 min at 56 °C, and tested for anti-HA antibodies levels using the HA inhibition (HI) assay with 0.5% turkey erythrocytes. The reciprocal of the largest serum dilution which thoroughly suppressed hemagglutination was deemed as the HI titers.

2.10. Statistical analysis

Graphpad Prism 5.0 software was used to analyze the data, and data were shown as mean ± SD. The probabilities of survival of mice infected with B/Memphis/20/96(R152) or B/Memphis/20/96(R152K) were assessed by Kaplan-Meier method, and the survival rates between the control and drug treatment groups were compared by log-rank test. Viral loads, lung weights, lung lesions, cytokines/chemokines levels and HI titers were compared by one-way analysis of variance (ANOVA) with Dunnett's test.

3. Results

3.1. T-705 treatment protected mice from lethal challenge with influenza B virus with or without reduced susceptibility to oseltamivir

We used wild-type B/Memphis/20/96 and its mutant, possessing

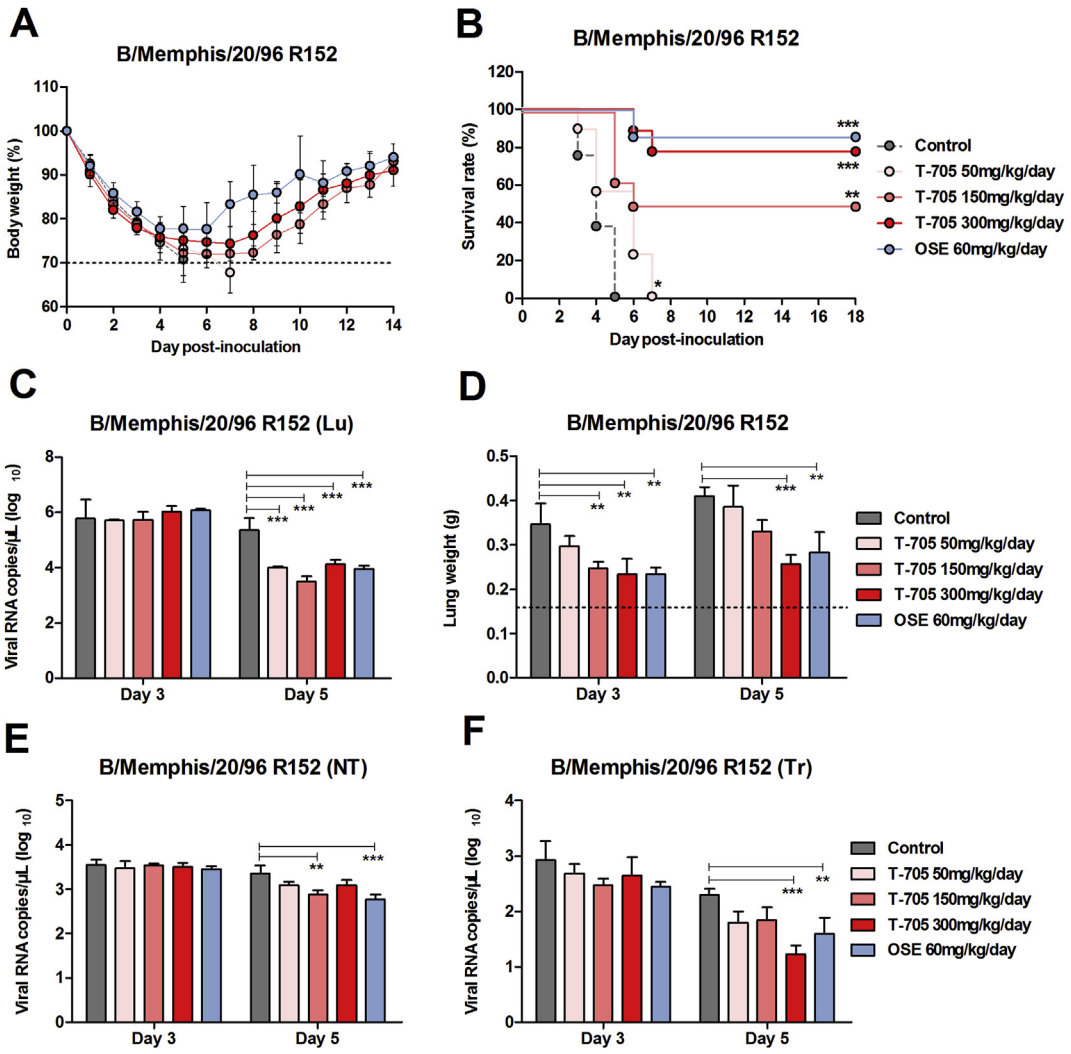

Fig. 1. Effect of T-705 and oseltamivir treatment on survival and viral load in mice infected with influenza B/Memphis/20/96(R152) virus. Eighteen mice per group were infected intranasally with B/Memphis/20/96(R152) virus (50 μl/mouse). Beginning at 2 hpi, T-705 at dosages of 50, 150 or 300 mg/kg/day or oseltamivir at the dosage of 60 mg/kg/day was orally administered twice daily for 5 days. Control animals orally received 200 μl of 0.4% CMC. Weight loss of mice was monitored for 14 dpi daily (A), and survival was observed daily to 18 dpi (B). On 3 and 5 dpi, NS1 gene copy numbers were detected by digital PCR in lungs (C), nasal turbinates (E) and tracheas (F) of mice (n = 3 per time point); and lung weight was recorded (D). The dotted line (D) represents mean lung weight in uninfected control mice. Differences between groups were analyzed by Kaplan-Meier method for survival and one-way ANOVA for viral loads and lung weights. *P < 0.05, **P < 0.01 and ***P < 0.001 versus control group. OSE indicates oseltamivir, Lu indicates lung, NT indicates nasal turbinate, Tr indicates trachea.

the arginine to lysine substitution at position 152 in NA, to estimate the efficacy of T-705. Prior to animal experiments, we tested the susceptibility of B/Memphis/20/96(R152) and B/Memphis/20/96(R152K) to T-705 and oseltamivir carboxylate in vitro and displayed the EC_{50} values in Table S1. The results showed that the susceptibility of B/Memphis/20/96(R152K) to T-705 was similar to that of B/Memphis/20/96(R152), while B/Memphis/20/96(R152K) is about 150 times less susceptible than B/Memphis/20/96(R152) to oseltamivir carboxylate.

The untreated control animals infected with B/Memphis/20/96(R152) lost weight and succumbed to infection at 3 to 5 dpi (Fig. 1A and B). Prevention of body-weight loss was dose-dependent in T-705 treatment groups, but without statistical difference (Fig. 1A). An obvious dose-dependent response to survival was achieved: higher dosages of T-705 related to higher survival rates (Fig. 1B). T-705 administration at 50 mg/kg/day provided no protection compared to the control group, while the two higher dosages of T-705 at 150 and 300 mg/kg/day led to 50% and 78% survival rates, respectively; and, oseltamivir treatment conferred better protection than all dosages of T-705 did (Fig. 1B).

To mice infected with B/Memphis/20/96(R152K) virus, the control group lost weight and succumbed to infection at 5 to 7 dpi (Fig. 2A and B). T-705 treatment at dosages of 150 or 300 mg/kg/day displayed slight reduction in body weight loss and exhibited less peak weight loss compared to control and oseltamivir-treated groups (Fig. 2A). Fewer days were needed for body weight recovering in T-705-treated animals, when compared to control group (Fig. 2A). An apparent dose-dependent response to survival was also observed in T-705-treated B/Memphis/20/96(R152K) infection group. Survival rate ranged from 22% to 89%, depending on the dosage (Fig. 2B). Mice administered with the lowest dosage of T-705 (50 mg/kg/day) had same survival rate of 22% with that of oseltamivir (60 mg/kg/day) (Fig. 2B). In conclusion, these results demonstrate that T-705 at doses ≥150 mg/kg/day can improve survival of mice challenged with oseltamivir-resistant influenza B virus.

3.2. T-705 treatment significantly decreased viral loads in mice infected with B/Memphis/20/96(R152) or B/Memphis/20/96(R152K)

In addition to survival rate, viral loads in the respiratory tract are also important in assessing the effect of T-705 on mice infected with influenza viruses. So, to determine the effect of T-705 treatment on

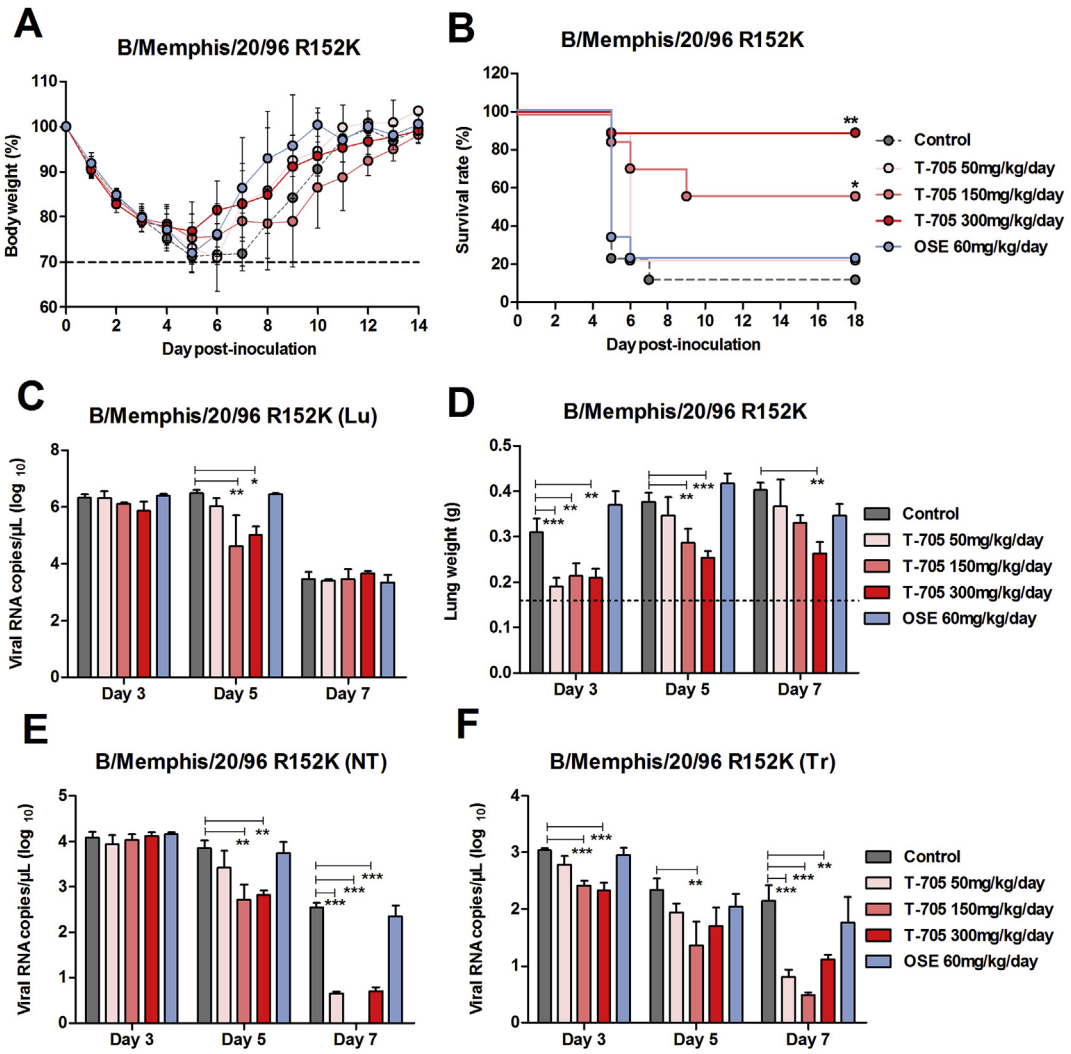

Fig. 2. Effect of T-705 and oseltamivir treatment on survival and viral load in mice infected with influenza B/Memphis/20/96(R152K) virus. Weight loss of mice was monitored for 14 dpi daily (A), and survival was observed daily to 18 dpi (B). On 3, 5, and 7 dpi, NS1 gene copy numbers were detected by digital PCR in lungs (C), nasal turbinates (E) and tracheas (F) of mice; and lung weight was recorded (D). The dotted line (D) represents mean lung weight in uninfected control mice. Statistical analysis was performed as described in Fig. 1.

viral loads at different time points, we measured nonstructural protein 1 (NS1) gene copy numbers of influenza virus in lungs, nasal turbinates and tracheas of mice at 3, 5, or 7 dpi, with digital PCR.

For B/Memphis/20/96(R152) virus infection, at 5 dpi, administration of T-705 to all test groups significantly reduced RNA copy numbers in lungs, when compared to those in controls (Fig. 1C). At 5 dpi, T-705 at the dosage of 150 mg/kg/day significantly decreased RNA copies in nasal turbinates; although 300 mg/kg/day dosage only exhibited slight efficacy (Fig. 1E). For tracheas, treatment of 300 mg/kg/day T-705 significantly reduced RNA copy numbers, at 5 dpi (Fig. 1F). As drug control, oseltamivir administration significantly decreased RNA copy numbers in all types of tissues (Fig. 1C, E and F).

In mice infected with B/Memphis/20/96(R152K) virus, gene copy numbers in lungs and nasal turbinates were significantly reduced by the two highest T-705 dosages, compared with that of controls, at 5 dpi (Fig. 2C and E). By day 7, treatment of T-705 at all dosages resulted in significantly lower gene copy numbers in nasal turbinates and tracheas compared to control group (Fig. 2E and F). For tracheas, 150 mg/kg/day T-705 significantly decreased RNA copy numbers at all time points compared to control group (Fig. 2F). In comparison, administration of oseltamivir had little effect on viral loads in all tissue samples of mice at any time points, compared to controls (Fig. 2C, E and F). Together, these results prove the capability of T-705 treatment to diminish viral loads in mice infected with oseltamivir-resistant influenza B virus.

3.3. T-705 treatment significantly reduced lung weight gain in mice infected with B/Memphis/20/96(R152) or B/Memphis/20/96(R152K)

Besides survival rate and viral load, the level of inflammation in the lungs of mice is essential in evaluating the effect of T-705 on mice infected with influenza virus, which can be displayed using three indicators, including lung weight, lung lesion, and cytokine/chemokine level.

Lung weight of mice infected with influenza virus usually increases over time owning to inflammation induced by virus infection (Fukao et al., 2019). For B/Memphis/20/96(R152) virus infection, treatment of mice with T-705 dose-dependently reduced the elevation of lung weight compared with control. At 3 and 5 dpi, this increase of lung weight was significantly suppressed by T-705 at 300 mg/kg/day and oseltamivir at 60 mg/kg/day compared with control (Fig. 1D).

In mice infected with B/Memphis/20/96(R152K) virus, at 3 dpi, T-705 at all dosages significantly reduced the elevation in lung weights compared with that of control and oseltamivir-treated mice. At 5 dpi, mice receiving T-705 at dosages of 150 or 300 mg/kg/day achieved lower lung weights compared with control and oseltamivir-treated mice (Fig. 2D). Overall, these data prove the capability of T-705 treatment to

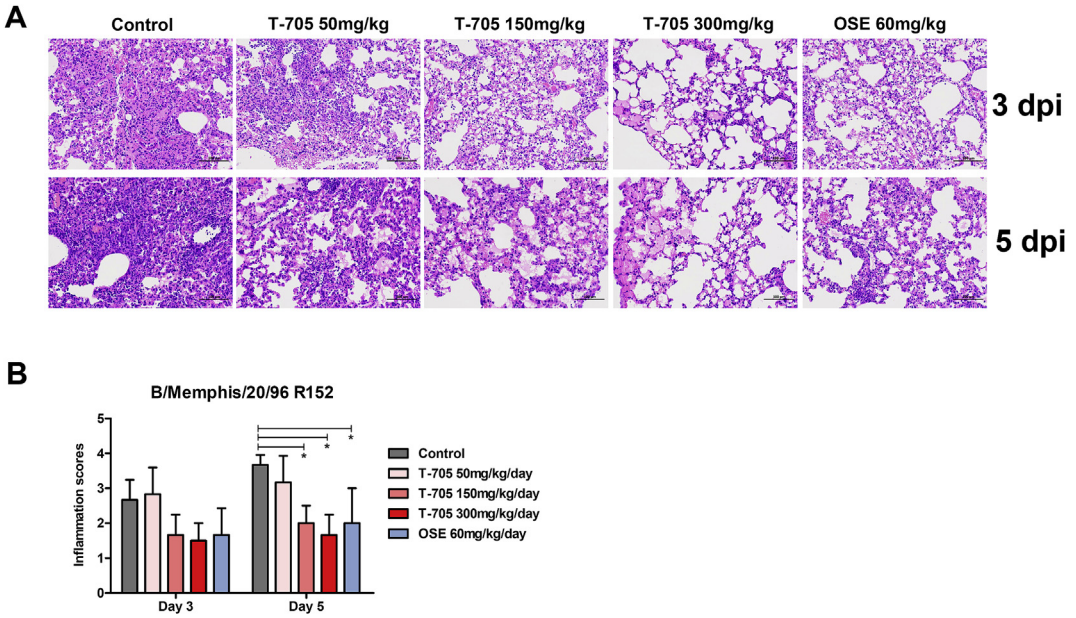

Fig. 3. Effect of T-705 and oseltamivir treatment on lung injury in mice infected with influenza B/Memphis/20/96(R152) virus. (A) The results of pulmonary pathology. At 3 and 5 dpi, lung tissue was fixed with 10% paraformaldehyde and H&E staining was carried out to estimate pathological changes (n = 3/group). Scale bars were 100 μm. (B) Scores of inflammation in lungs after B/Memphis/20/96(R152) virus challenge. The values ranged from 0 (normal) to 4 (severe). 0, normal; 1, increased thickness of the inter-alveolar septa by oedema and cell infiltration; 2, increased thickness of the inter-alveolar septa with the presence of luminal cell infiltration; 3, abundant luminal cell infiltration; and 4, inflammatory patches formed (Zhang et al., 2019). One-way ANOVA was used to compare the scores. *P < 0.05 versus control group.

curb lung weight gain in mice infected with oseltamivir-resistant influenza B virus.

3.4. T-705 treatment significantly reduced lung lesions in mice

To assess lung injury, we examined lung sections to detect the changes in lung morphology of infected mice at 3, 5 and 7 dpi. Results showed lung lesions were reduced upon T-705 administration in most mice (Figs. 3 and 4).

For both B/Memphis/20/96(R152) and B/Memphis/20/96(R152K) virus infection, control and 50 mg/kg/day T-705-treated mice presented severe inflammation with alveolar inflammatory patches; and lung injuries were observed in most of the lung lobes of all mice (Figs. 3 and 4). When compared with control groups, mice receiving T-705 at

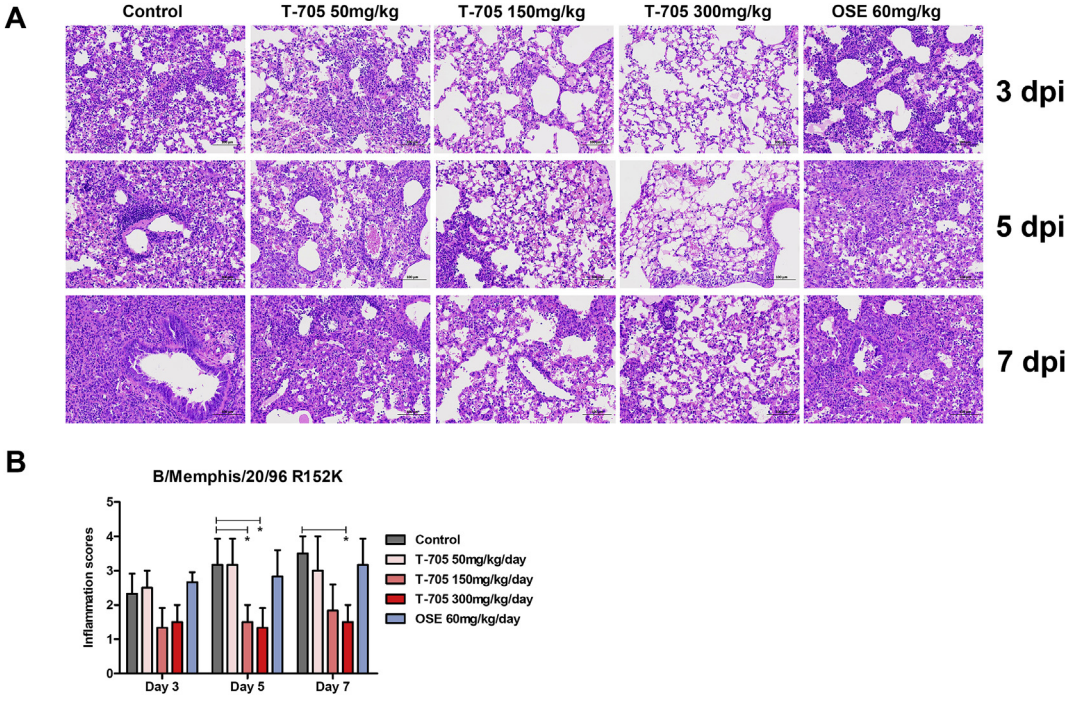

Fig. 4. Effect of T-705 and oseltamivir treatment on lung damage in mice infected with influenza B/Memphis/20/96(R152K) virus. (A) The results of pulmonary pathology. At 3, 5, and 7 dpi, lung tissue was fixed with 10% paraformaldehyde and H&E staining was performed to assess pathological changes (n = 3/group). Scale bars were 100 μm. (B) Scores of inflammation in lungs after B/Memphis/20/96(R152K) virus challenge.

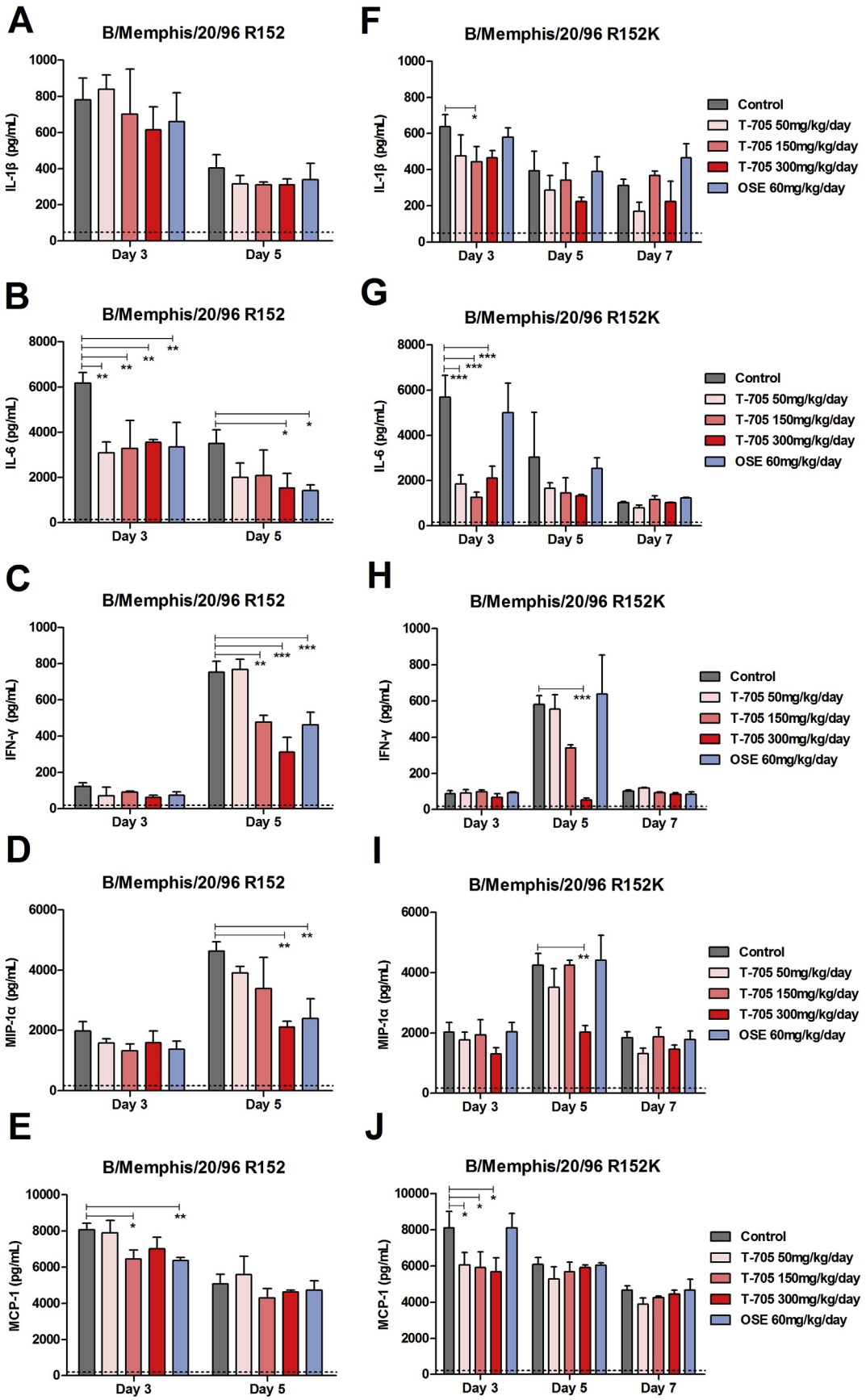

Fig. 5. Effect of T-705 and oseltamivir treatment on cytokine/chemokine levels in the lungs of mice infected with influenza B/Memphis/20/96(R152) or B/Memphis/20/96(R152K) virus. On 3, 5 and 7 dpi, ELISA kits were used to measure the concentrations of IL-1β (A and F), IL-6 (B and G), IFN-γ (C and H), MIP-1α (D and I) and MCP-1 (E and J) in the lungs of infected mice. The dotted line represents the mean cytokine/chemokine level in uninfected control mice. One-way ANOVA was used to perform statistical analysis. *P < 0.05, **P < 0.01 and ***P < 0.001 versus control group.

dosages of 150 and 300 mg/kg/day displayed slight to moderate lung infiltration. Furthermore, T-705 treatment showed a dose-dependent effect on reducing lung lesions at most time points (Figs. 3 and 4). In addition, oseltamivir treatment showed similar efficacy to T-705 at dosages of 150 or 300 mg/kg/day against B/Memphis/20/96(R152) (Fig. 3), but negligible efficacy against B/Memphis/20/96(R152K) (Fig. 4). These data prove that T-705 treatment curbs lung pathology in mice infected with oseltamivir-resistant influenza B virus.

3.5. T-705 treatment reduced the levels of cytokines/chemokines in mice

To estimate disease severity following inoculation, we determined the levels of cytokines/chemokines in the lungs of infected mice at 3, 5 and 7 dpi.

For mice infected with B/Memphis/20/96(R152) virus, at 3 dpi, T-705 and oseltamivir administration led to lower levels of IL-6 and MCP-1 compared with control groups (Fig. 5B and E); at 5 dpi, T-705 at 300 mg/kg/day and oseltamivir at 60 mg/kg/day significantly reduced the levels of IL-6, IFN-γ and MIP-1α compared with control groups (Fig. 5B, C and D). The increase of IL-1β was slightly diminished with T-705 or oseltamivir administration without statistical differences (Fig. 5A).

For B/Memphis/20/96(R152K) virus infections, by day 3, T-705 treatment (all dosages) resulted in lower levels of IL-6 and MCP-1 compared with control and oseltamivir-treated groups (Fig. 5G and J); by day 5, the increase in IFN-γ and MIP-1α levels was significantly diminished upon T-705 treatment at the dosage of 300 mg/kg/day, compared to control and oseltamivir-treated groups (Fig. 5H and I). Compared with control groups, 150 mg/kg/day T-705 significantly decreased the levels of IL-1β (Fig. 5F). Taken together, these results displayed that T-705 treatment could significantly reduce the increase of cytokines/chemokines in the lungs of mice challenged with oseltamivir-resistant influenza B virus.

3.6. The emergence of resistant mutants was not observed after T-705 administration in vivo in this study

The emergence of drug-resistant mutants is always worthy of concern. Published data showed that clinical administration of NAIs and Baloxavir (an RdRp inhibitor) can lead to the emergence of resistance (Gubareva and Fry, 2020; Jones et al., 2017; Takashita et al., 2019; Webster and Govorkova, 2014). Thus, to demonstrate whether resistant viruses appeared under T-705 treatment in mice, 24 isolates of influenza viruses were recovered from the lungs of mice treated with T-705 for 5 days. We determined the EC_{50} of T-705 to protect MDCK cells from influenza virus infections, using a cell viability assay. The EC_{50} values of T-705 to viruses recovered from the lungs of control animals was 3.88 μM for R152 and 8.93 μM for R152K. Overall, we measured only slight change (0.84- to 1.24-fold) in EC_{50} values for viruses recovered from T-705-treated mice, compared with control groups (Table 1). Together, phenotypic analysis showed that T-705 treatment did not easily lead to the emergence of drug resistant mutants of influenza virus, which is consistent with previously published results (Baranovich et al., 2013; Pascua et al., 2019; Takashita et al., 2016).

Table 1
T-705 susceptibility of influenza B viruses recovered from lung homogenates.

Group (mg/kg/day)	B/Memphis/20/96 R152		B/Memphis/20/96 R152K	
	mean EC_{50} ± SD (μM)	fold change	mean EC_{50} ± SD (μM)	fold change
Control	3.88 ± 0.64	NA	8.93 ± 1.92	NA
50	4.50 ± 1.06	1.16	7.49 ± 1.67	0.84
150	4.82 ± 1.71	1.24	9.00 ± 1.92	1.01
300	3.92 ± 0.60	1.01	8.86 ± 1.88	0.99

NA, not applicable.

3.7. T-705 administration does not hinder the humoral immune response in mice infected with B/Memphis/20/96(R152) or B/Memphis/20/96(R152K)

We further tested the humoral immune response of surviving mice, which is an important indicator of the body's resistance to viral infections. To assess the effect of T-705 administration on the induction of humoral immunity following influenza B/Memphis/20/96(R152) and B/Memphis/20/96(R152K) virus infections, we detected the serum HI titers on day 21. HI titers of ≥40 were deemed as antibody positive (Zhu et al., 2019). Upon virus infection, all of the surviving mice had HI titers of > 40 (Fig. 6). We noticed a similar humoral immune response of B/Memphis/20/96(R152) and B/Memphis/20/96(R152K) virus infections following T-705 treatment (Fig. 6). In short, T-705 treatment does not significantly inhibit the production of anti-HA antibody.

4. Discussion

Favipiravir, also known as T-705, has broad-spectrum inhibitory activity against various RNA viruses, including influenza virus, measles virus (Jochmans et al., 2016), Rift Valley fever virus (Scharton et al., 2014), Ebola virus (Bai et al., 2016; McCarthy et al., 2016), Zika virus (Kim et al., 2018), and chikungunya virus (Delang et al., 2014) in vitro and in animal models. In this study, we estimated the efficacy of T-705 against influenza B virus and its oseltamivir-resistant variant, and determined the potential of the emergence of T-705-resistant mutants in a lethal mouse model.

Seasonal influenza viruses, including influenza B virus, mainly infect the upper respiratory tract, but in some high-risk groups, such as immunodeficiency patients, influenza infection can cause severe complications such as pneumonia (Ison, 2013). Our research showed that T-705 not only reduced the viral load in the upper respiratory tract, but also reduced the viral load in the lungs. Although infectious titers (e.g., $TCID_{50}$) is more commonly used to assess the level of virus replication, determining viral loads using digital PCR can also reflect the level of virus replication to some extent. In addition, T-705 could decrease lung cytokine storms and lung damage. Although lung sections of uninfected mice were missing which was a shortcoming in our study, we mainly focus on the differences between drug-treated mice and untreated, infected control mice. As a treatment control, in mice infected with B/Memphis/20/96(R152) virus, oseltamivir treatment showed similar efficacy to T-705 at 300 mg/kg/day, while had negligible efficacy against B/Memphis/20/96(R152K) infected mice.

Moreover, the emergence of drug-resistant mutants during drug treatment is always worthy of concern. Although no case of resistance to T-705 has been reported clinically, a K229R mutation in the PB1 subunit of the influenza virus conferring resistance to T-705 in vitro has been reported (Goldhill et al., 2018). So, phenotypic assay was performed to determine the EC_{50} of T-705 to viruses recovered from lung homogenates of mice. Compared with control groups, increase up to 1.24-fold in EC_{50} values was determined for viruses recovered from T-705-treated mice (Table 1), which proved that viruses under T-705 treatment in vivo were still susceptible to T-705. One limitation of this study is that we did not conduct sequence analysis based on NGS to further conclude that resistant mutants did not emerge. Additionally, no T-705-resistant viruses were isolated, probably because the model used in this study did not favor the emergence of resistance. For example, only one passage of virus was performed in mice; mice were not treated with increasing doses of T-705; and T-705 treatment lasted only 5 days which was too short. Furthermore, the EC_{50} values of T-705 to viruses recovered from the lungs in our study were 3.88–4.82 μM for R152 and 7.49–9.00 μM for R152K, which were different from 1.21 μM for R152 and 0.57 μM for R152K published by Sleeman et al. (2010). This difference may be due to the different methods used when determining the

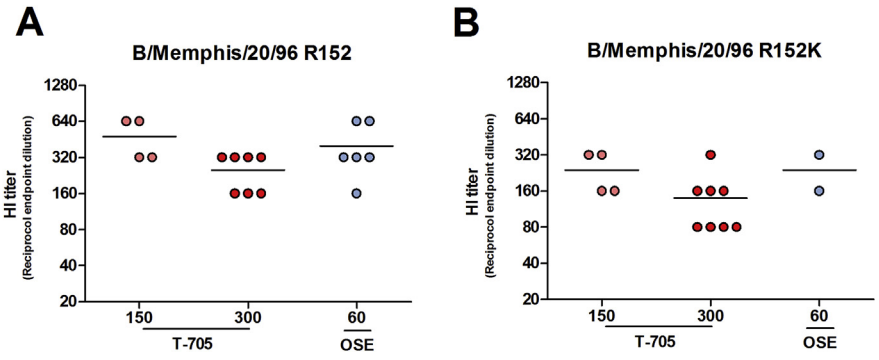

Fig. 6. Effect of T-705 and oseltamivir treatment on the humoral immune response in mice infected with influenza B/Memphis/20/96(R152) or B/Memphis/20/96(R152K) virus. On day 21 post infection, the HI titers were determined from the remaining mice challenged with B/Memphis/20/96(R152) (A) or B/Memphis/20/96(R152K) viruses (B). The reciprocal values were used to represent HI titers. The HI titers between groups were compared by one-way ANOVA.

EC_{50}.

Although previous studies have proven the efficacy of T-705 against oseltamivir-resistant pandemic influenza A(H1N1) viruses (Manicassamy et al., 2014; Smee et al., 2013) and oseltamivir-resistant highly pathogenic H5N1 and H7N9 influenza viruses (Kiso et al., 2010; Zhang et al., 2014) in mice models, this is the first time to study the efficacy of T-705 in mice challenged with oseltamivir-resistant influenza B viruses. Moreover, not only can it be used for severe influenza B infection, T-705 could have been an alternative in previously reported cases infected with high percentage of NAI-resistant H7N9 strains. Additionally, previous studies demonstrated the efficacy of T-705 combination with NAIs in mice infected with pandemic influenza A(H1N1) viruses (Baz et al., 2018; Takahashi et al., 2003; Tarbet et al., 2012), influenza A(H3N2) viruses (Smee et al., 2009) and highly pathogenic influenza A(H5N1) viruses (Marathe et al., 2016). Thus, it is important to demonstrate if T-705 combination with NAIs could display more potent inhibitory efficacy against influenza B viruses in vivo in future research.

In conclusion, our data indicate that T-705 can be effective against influenza B viruses, and can be a promising alternative antiviral to treat severe oseltamivir-resistant influenza B virus infections in patients.

CRediT authorship contribution statement

Qiong-Qiong Fang: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing. **Wei-Juan Huang:** Project administration, Resources. **Xi-Yan Li:** Project administration, Resources. **Yan-Hui Cheng:** Project administration, Resources. **Min-Ju Tan:** Project administration, Resources. **Jia Liu:** Project administration, Resources. **He-Jiang Wei:** Project administration, Resources. **Yao Meng:** Project administration, Resources. **Da-Yan Wang:** Conceptualization, Formal analysis, Validation, Resources, Writing - review & editing, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2020.02.005.

Supplementary Table 1
Susceptibility of virus inoculum to antivirals.

Virus	Cell viability EC_{50} ± SD (μM)	NA inhibition EC_{50} ± SD (nM)
	T-705	Oseltamivir carboxylate
B/Memphis/20/96 R152	4.33 ± 0.92	6.45 ± 0.26
B/Memphis/20/96 R152K	6.45 ± 0.44	995 ± 40.5

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