Optimizing T-705 (favipiravir) treatment of severe influenza B virus infection in the immunocompromised mouse model

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Background: Influenza B virus infections remain insufficiently studied and antiviral management in immunocompromised patients is not well defined. The treatment regimens for these high-risk patients, which have elevated risk of severe disease-associated complications, require optimization and can be partly addressed via animal models.

Methods: We examined the efficacy of monotherapy with the RNA-dependent RNA polymerase inhibitor T-705 (favipiravir) in protecting genetically modified, permanently immunocompromised BALB *scid* mice against lethal infection with B/Brisbane/60/2008 (BR/08) virus. Beginning at 24 h post-infection, BALB *scid* mice received oral T-705 twice daily (10, 50 or 250 mg/kg/day) for 5 or 10 days.

Results: T-705 had a dose-dependent effect on survival after BR/08 challenge, resulting in 100% protection at the highest dosages. With the 5 day regimens, dosages of 50 or 250 mg/kg/day reduced the peak lung viral titres within the treatment window, but could not efficiently clear the virus after completion of treatment. With the 10 day regimens, dosages of 50 or 250 mg/kg/day significantly suppressed virus replication in the lungs, particularly at 45 days post-infection, limiting viral spread and pulmonary pathology. No T-705 regimen decreased virus growth in the nasal turbinates of mice, which potentially contributed to the viral dynamics in the lungs. The susceptibility of influenza B viruses isolated from T-705-treated mice remained comparable to that of viruses from untreated control animals.

Conclusions: T-705 treatment is efficacious against lethal challenge with BR/08 virus in immunocompromised mice. The antiviral benefit was greatest when longer T-705 treatment was combined with higher dosages.

Introduction

Immunocompromised individuals are at increased risk of influenza virus infection. Their impaired immunity can enable infections to progress to the lower respiratory tract (LRT) and influenza-attributed disease burden is often associated with high morbidity, mortality and subsequent complications.^{1–4} Infections of immunocompromised patients are defined by persistent virus replication requiring prolonged drug treatment, which contributes to the development of antiviral drug resistance. Furthermore, their response to vaccination is relatively poor.^{1,2,4} During annual seasonal influenza epidemics, influenza B viruses co-circulate with influenza A viruses, causing clinically indistinguishable respiratory diseases.^{5–7} Although data on the epidemiology and disease burden remain limited, there is evidence suggesting a high incidence of complications and death due to influenza B in young children and elderly adults.^{6,8–10} Influenza B virus infections account for 29% of influenza-attributable deaths in an average season, but

this percentage could reach 95% in years with high virus circulation.⁹ Moreover, several extrapulmonary complications (e.g. myositis, neurological or gastrointestinal symptoms) that often affect immunocompromised children have been linked to influenza B virus infections.¹¹ Neuraminidase inhibitors (NAIs) are recommended for treatment and prophylaxis of influenza B virus infections worldwide;¹¹ however, clinical studies have shown their reduced efficacy, even in immunocompetent patients.^{12,13}

The partial efficacy of NAIs against influenza B virus infections has been demonstrated in immunocompromised mouse models.^{14,15} In genetically modified, permanent severely combined immunodeficient BALB/c (BALB *scid*) mice, the NAI peramivir rescued animals from lethal challenge, but could not efficiently inhibit prolonged virus persistence.¹⁵ In pharmacologically immunosuppressed BALB/c mice, the NAI oseltamivir also improved survival and reduced pulmonary virus spread, but did not provide complete protection or decrease the virus titres.¹⁴ Hence, evaluation of the

efficacy of next-generation virus inhibitors in antiviral management of influenza among immunocompromised hosts is required.

We assessed the efficacy of the RNA-dependent RNA polymerase inhibitor T-705 (favipiravir) in protecting BALB *scid* mice against lethal influenza B virus challenge. T-705 was approved for restricted use and pandemic stockpiling in Japan in 2014 and is undergoing Phase III clinical trials in the USA.^{16–18} T-705 is recommended for 5 day treatments in immunocompetent patients at the initial loading dosage of 1800 mg/kg twice daily, with maintenance dosages of 600–800 mg/kg twice daily on days 2–5.^{17,18} In recent Phase II studies in the USA, regimens have been extended to 10 days for individuals with severe influenza.¹⁹ Although showing *in vitro* activity,^{20,21} T-705 efficacy against influenza B viruses has never been examined experimentally *in vivo*. This study investigated the therapeutic value of T-705 in treating severe influenza B virus infection in an immunocompromised murine model. We focused on the appropriate range of dosages and duration of treatment needed to achieve optimal efficacy. We show that both a high dosage (≥ 50 mg/kg/day) and treatment for 10 days promotes a better outcome in mice with impaired immune responses against severe influenza B virus infection.

Materials and methods

Ethics statement

All animal experiments were conducted in a biosafety level 2 facility at St. Jude Children's Research Hospital (Memphis, TN, USA) after approval by the Institutional Animal Care and Use Committee and were performed in accordance with the applicable laws and guidelines of the NIH and the Animal Welfare Act.

Influenza B virus, cells and T-705

The influenza B/Brisbane/60/2008 (BR/08, Victoria lineage) virus was obtained from the Influenza Division of the CDC. Virus stock was grown in the allantoic cavities of 10-day-old embryonated chicken eggs for 72 h at 33°C and stored at –80°C until use. Titres in virus stocks and mouse tissues (nasal turbinates and lungs) were determined by TCID₅₀ assays,²² using Madin–Darby canine kidney (MDCK) cells (ATCC, Manassas, VA, USA) maintained in EMEM containing 5% FBS. T-705 working solutions were freshly prepared by either mixing with ORA-Plus suspending vehicle (Perrigo, Minneapolis, MN, USA) for mouse administrations or diluted in infection medium for phenotypic susceptibility assays.

Assessment of T-705 efficacy in mice

The pathogenicity of influenza BR/08 virus in immunocompetent BALB/c and immunocompromised BALB *scid* mice (The Jackson Laboratory, Bar Harbor, ME, USA) was validated by inoculating 6-week-old female mice (30 µL/mouse), under light isoflurane anaesthesia, with 10^3 – 10^6 and 10^2 – 10^5 TCID₅₀ virus doses, respectively (Figure S1, available as [Supplementary data](#) at JAC Online). The virus doses that caused >60% lethality in these genetically matched mouse strains (10^6 TCID₅₀ for BALB/c and 10^5 TCID₅₀ for BALB *scid*) were used for assessing T-705 efficacy.

At 24 h post-infection (hpi), mice were treated by oral gavage twice daily (12 h intervals) for 5 or 10 days with 10, 50 or 250 mg/kg/day of T-705 (100 µL/mouse). Control animals received 100 µL of ORA-Plus twice daily for 5 or 10 days. Animals were monitored for morbidity and mortality for 21 days post inoculation (dpi) (immunocompetent mice) or 45 dpi (immunocompromised mice). Mice were observed daily for clinical signs, weight loss and survival ($n = 8$ /group). Mice that lost >30% of their initial body weight were euthanized. Nasal turbinate and lung tissues were

harvested at 3, 5 and 7 dpi from BALB/c mice, 5, 10, 15, 30 and 45 dpi from BALB *scid* mice treated for 5 days, and 10, 15, 20, 30 and 45 dpi from BALB *scid* mice treated for 10 days ($n = 3$ /day/group). Tissue samples were rinsed with sterile PBS, homogenized and suspended in 1 mL of ice-cold PBS. Cellular debris was removed by centrifugation at 2000 rpm for 10 min.

Lung histopathology and immunohistochemistry

BALB/c (at 5 and 7 dpi) and BALB *scid* (at 5, 10 and 45 dpi for 5 day treatments and at 10, 15 and 45 dpi for 10 day treatments) mice ($n = 3$ /group) were subjected to whole-lung perfusion with 10% neutral-buffered formalin solution (Thermo Fisher Scientific, Waltham, MA, USA). Lungs were processed routinely and embedded in paraffin, then sections were subjected to haematoxylin–eosin and immunohistochemical staining with polyclonal goat antiserum against the HA glycoprotein of B/Florida/04/2006. Systematic scoring of acute lung injury by histology was performed in a blinded fashion.^{14,15}

Susceptibility to T-705 *in vitro*

MDCK cells were inoculated with paired turbinate and lung homogenate samples obtained at 10 dpi (from mice treated for 5 days) or 20 dpi (from mice treated for 10 days) and were incubated at 33°C for 72 h. The susceptibility of recovered viruses to T-705 was assessed in MDCK cells (1.5×10^4 cells/well in 96-well flat-bottom plates) inoculated with virus at an moi of 0.01 TCID₅₀/cell. Cell viability in the presence of T-705 (0.14–300 µM) at 72 hpi was measured with a CellTiter-Glo luminescent assay (Promega, Madison, WI, USA), for calculating EC₅₀.²³

Statistical analysis

Virus titres and EC₅₀s were compared by one- or two-way analysis of variance (ANOVA) with Bonferroni's multiple comparison post-test (GraphPad Prism 7.0, La Jolla, CA, USA). The Kaplan–Meier method was used to estimate the probability of survival for BR/08-inoculated mice and the log-rank test was used to compare survival rates in the control and treatment groups.

Results

Efficacy of T-705 against lethal BR/08 infection in immunocompetent mice

To assess the therapeutic efficacy of T-705 against lethal BR/08 infection in immunocompetent mice, their morbidity and survival were monitored. The control untreated BALB/c mice became scruffy and hunched, lost weight and developed respiratory distress and, by 8 dpi, only 12.5% of these mice survived (Figure 1a and b and Table S1). The highest survival rate achieved in treated mice was 75%, with 250 mg/kg/day, followed by 50%, with 50 mg/kg/day. Administration of T-705 at 10 mg/kg/day did not improve survival in these animals, as compared with control mice (Figure 1b).

T-705 administration decreased virus replication in the respiratory tract of mice in a dose-dependent manner; this was more evident in the LRT (the lungs) than in the upper respiratory tract (URT) (the turbinates) (Figure 1c and d and Table S2). Virus replication in control animals and in mice treated with 10 mg/kg/day of T-705 lasted for 5–7 dpi in the URT and LRT. In contrast, treatments with 50 and 250 mg/kg/day were similarly efficient at limiting virus replication in the LRT, with titres detected only at 3 dpi (Figure 1d). Only mice treated with 250 mg/kg/day cleared the virus from their

Figure 1. Effect of 5 day T-705 treatment on morbidity and survival of BR/08 virus-infected immunocompetent mice and on virus replication therein. BALB/c mice were anaesthetized with isoflurane and inoculated intranasally with 10^6 TCID₅₀ of BR/08 virus (30 μ L/mouse). T-705 was administered by oral gavage twice daily for 5 days at a dosage of 10 (orange lines and bars), 50 (blue lines and bars) or 250 (green lines and bars) mg/kg/day (100 μ L/mouse) starting at 24 hpi. Control untreated mice (black lines and bars) were gavaged twice daily for 5 days with 100 μ L of ORA-Plus suspending vehicle on the same schedule. Weight loss (a) and survival (b) of mice ($n = 8$ /group) were monitored daily to 21 dpi. The horizontal broken line in (a) indicates the endpoint for mortality (30% loss of initial weight). Viral titres in the URT and LRT were determined in nasal turbinates (c) and lung tissue samples (d) at 3, 5 and 7 dpi. Error bars in (c) and (d) represent SD of viral titres obtained from 3 animals. The probabilities of survival were determined by Kaplan–Meier and log-rank tests and virus titres were analysed by two-way ANOVA with Bonferroni's multiple comparison post-test. Statistical analyses are shown in [Tables S1 and S2](#). * $P < 0.05$ relative to control group, # $P < 0.05$ relative to 10 mg/kg/day group, + $P < 0.05$ relative to 50 mg/kg/day group and + $P < 0.05$ relative to 250 mg/kg/day group.

Table 1. Susceptibility to T-705 of influenza B viruses recovered from nasal turbinate and lung tissue samples^a

Treatment group (mg/kg/day)	5 day treatment				10 day treatment			
	turbinate		lungs		turbinate		lungs	
	mean EC ₅₀ ± SD (μM)	fold change	mean EC ₅₀ ± SD (μM)	fold change	mean EC ₅₀ ± SD (μM)	fold change	mean EC ₅₀ ± SD (μM)	fold change
10	49.8 ± 5.6	1.3	50.1 ± 3.2	0.9	24.9 ± 3.2	0.7	47.8 ± 8.4	1.2
50	57.9 ± 6.4	1.5	45.5 ± 4.4	0.8	45.3 ± 4.1	1.2	47.9 ± 6.1	1.2
250	51.0 ± 3.0	1.3	42.3 ± 12.2	0.8	41.1 ± 7.4	1.1	51.4 ± 3.9	1.3
Control	38.1 ± 9.7	NA	54.9 ± 2.4	NA	36.8 ± 5.0	NA	39.7 ± 4.3	NA

NA, not applicable.

^aCell viability assays were performed in tissue samples collected at 10 and 20 dpi for 5 and 10 day treatments, respectively.

Figure 2. Effect of T-705 regimens on morbidity and survival of BR/08 virus-infected immunocompromised mice. BALB *scid* mice were anaesthetized with isoflurane and inoculated intranasally with 10^5 TCID₅₀ of BR/08 virus (30 μ L/mouse). T-705 was administered by oral gavage twice daily for 5 (a and b) or 10 (c and d) days at a dosage of 10 (orange lines and bars), 50 (blue lines and bars) or 250 (green lines and bars) mg/kg/day (100 μ L/mouse) starting at 24 hpi. Control untreated mice (black lines and bars) were gavaged twice daily for 5 or 10 days with 100 μ L of ORA-Plus suspending vehicle. Weight loss (a and c) and survival (b and d) of mice ($n = 8$ /group) were monitored daily to 45 dpi. The horizontal broken line in (a) and (c) indicates the endpoint for mortality (30% loss of initial weight). The probabilities for survival were determined by Kaplan–Meier and log-rank tests. Statistical analyses are shown in Table S1. + $P < 0.05$ relative to 50 mg/kg/day group and $\pm P < 0.05$ relative to 250 mg/kg/day group.

URT at 7 dpi (Figure 1c). Thus, T-705 administered at ≥ 50 mg/kg/day could suppress BR/08 replication and prevent death in BALB/c mice.

Efficacy of T-705 against lethal BR/08 infection in immunocompromised mice

The therapeutic efficacy of T-705 against lethal influenza B virus infection was examined in BALB *scid* mice treated for 5 days (as in immunocompetent mice) or 10 days (Figure 2). With the 5 day regimen, higher dosages (50 or 250 mg/kg/day) protected 100% of inoculated animals, with no signs of morbidity during (at 2–6 dpi) or after completing treatments (> 7 dpi) (Figure 2a and b and Table S1). Survival rate in the 10 mg/kg/day group was similar to that seen in the control group with 62.5% (5/8) surviving infection. Thus, 5 day treatment protected immunocompromised hosts against lethal BR/08 challenge, but only treatment with ≥ 50 mg/kg/day resulted in 100% survival.

With the 10 day regimens, the weight losses in control mice and those treated with 10 mg/kg/day were similar, with 25% and 37.5% survival rates, respectively (Figure 2c and d and Table S1). Although one animal in the 50 mg/kg/day-treated group lost $> 30\%$ of its initial body weight (87.5% survival), all of the 250 mg/kg/day-treated mice survived lethal infection with no

signs of morbidity. Thus, the higher T-705 dosages, particularly 250 mg/kg/day, were consistently most beneficial with respect to morbidity and survival for BALB *scid* mice treated for 5 or 10 days.

Effect of T-705 treatment on virus replication in immunocompromised mice

To correlate the improved survival rates with the regulation of virus replication, viral titres were examined in the URT and LRT of T-705-treated immunocompromised mice. With the 5 day regimens, virus replication persisted to 45 dpi in untreated control mice; it did not change significantly over time in the turbinates, but gradually decreased in the lungs (Figure 3a and b). In the T-705-treated groups, administration of 250 mg/kg/day significantly lowered virus titres in the URT at 5 dpi ($P < 0.05$), but they rebounded and trended comparably with those in the other drug-treated groups at later timepoints after completion of treatments (Figure 3a and Table S3). Consistent with the results in BALB/c mice, the effect of T-705 treatment was more pronounced in the LRT of BALB *scid* mice (Figure 3b). Lung titres in the 10 mg/kg/day group were comparable to those in controls during drug administration and were significantly higher ($P < 0.05$) than those in the high-dosage groups (Figure 3b and Table S3). At 5 dpi, no viral titres were

Figure 3. Effect of T-705 regimens on virus replication in the respiratory tract of BR/08 virus-infected immunocompromised mice. Virus titres were determined at 5, 10, 15, 30 and 45 dpi in BALB *scid* mice treated for 5 days (a and b) or at 10, 15, 20, 30 and 45 dpi in BALB *scid* mice treated for 10 days (c and d). Virus titrations from samples obtained from nasal turbinates (a and c) and lung tissues (b and d) were performed by TCID₅₀ assays on MDCK cells ($n = 3/\text{group}/\text{timepoint}$). Error bars represent SD of viral titres obtained from 3 animals. §Detection in one available animal. †Some animals died before respiratory samples were collected. Virus titres were analysed by one-way ANOVA with Bonferroni's multiple comparison post-test. Statistical analyses are shown in [Tables S3](#) and [S4](#). * $P < 0.05$ relative to control group, # $P < 0.05$ relative to 10 mg/kg/day group, + $P < 0.05$ relative to 50 mg/kg/day group and $P < 0.05$ relative to 250 mg/kg/day.

detected in the LRT of mice treated with 250 mg/kg/day, but titres substantially increased at succeeding timepoints, suggesting a relapse of replication after treatment completion (≥ 10 dpi). None of the 5 day T-705 regimens suppressed LRT virus replication completely. Thus, 5 day treatments insufficiently halted virus replication in these immunocompromised animals, despite decreasing morbidity and mortality (Figure 2).

With the 10 day regimens, the control mice succumbed to infection, with none remaining for virus titration by 30 dpi (Figure 3c). T-705 did not significantly alter virus replication in the URT, except at 45 dpi in the 50 mg/kg/day group (Figure 3c and [Table S4](#)). At 10 dpi, lung viral titres in mice treated with 50 or 250 mg/kg/day were reduced significantly ($P < 0.05$) compared with those in controls and 10 mg/kg/day-treated mice (Figure 3d and [Table S4](#)). No mice treated with 10 mg/kg/day had detectable lung viral titres at 20 dpi, but viral replication restarted thereafter. The 250 mg/kg/day group became positive for virus only at 20 dpi; elevated titres were also noted in the 50 mg/kg/day group. However, lung viral titres were undetectable in both high-dosage treatment groups at 45 dpi.

Overall, prolonged T-705 administration reduced and regulated virus titres in the LRT of immunocompromised mice, particularly at

higher dosages during the treatment window. Virus replication was also less robust with 10 day than 5 day regimens (Figure 3 and [Table S5](#)). Apparently, viral replication in the LRT gradually recovered from antiviral suppression, even in mice treated with 5 day or 10 day high-dosage regimens when T-705 treatment was withdrawn. However, the growth rates could not approach those in control and 10 mg/kg/day-treated mice.

Histological changes in BR/08-infected mice

In BALB/c mice, the lungs of BR/08-infected controls at 5 dpi exhibited prominent perivascular and intra-alveolar inflammation with extensive viral antigen, denoting widespread active infection (Figure 4a). T-705 treatment significantly reduced the extent and severity of virus infection in the lungs, particularly in the 250 mg/kg/day group (Figure 4b and c). At 7 dpi, small areas of antigen-positive lung parenchyma (red-shaded areas) were still evident in control mice and pulmonary lesions involved entire lung lobes (Figure 4d). In contrast, there was no evidence of active virus replication in the lungs of either treatment group and the extent of pulmonary lesions was markedly reduced (Figure 4e and f). Inflammatory cell infiltrates were notably increased in drug-treated

Figure 4. Histopathological findings in BR/08 virus-infected immunocompetent and immunocompromised mice treated with different T-705 regimens. BALB/c and BALB *scid* mice were inoculated intranasally with 10⁵ or 10⁵ TCID₅₀ of BR/08 virus (30 µL/mouse), respectively, and treated with T-705 at a dosage of 50 or 250 mg/kg/day twice daily (100 µL/mouse) for 5 or 10 days starting 24 hpi. Control mice (0 mg/kg/day) were treated twice daily for 5 or 10 days with 100 µL of ORA-Plus suspending vehicle. The panels show the extent of virus infection and histopathology caused by the BR/08 virus in the lungs of immunocompetent BALB/c and immunocompromised BALB *scid* mice evaluated at the indicated timepoints (*n* = 3/group/timepoint). A representative section is shown for each treatment group. For histomorphometry, the extent of virus spread was quantified by first capturing digital images of whole-lung sections stained for viral antigen by using an Aperio ScanScope XT Slide Scanner (Aperio

BALB/c mice, as compared with controls, suggesting that suppression of virus infection facilitated efficient pulmonary inflammatory response. Overall, high-dosage T-705 treatments significantly attenuated pulmonary damage and virus spread in BALB/c mice.

In immunocompromised mice, beneficial dosage-related effects due to T-705 were noted in both 5 day and 10 day regimens; the most profound therapeutic effects were seen with the highest dosage/longer duration regimens. In 5 day treatments, clear dosage-related declines in the extent of both antigen-positive (red-shaded areas) and -negative (yellow-shaded areas) pulmonary lesions were noted at 5 and 10 dpi, which were significantly smaller ($P < 0.05$) in 250 mg/kg/day-treated mice (Figure 4g–l). At 45 dpi, areas of active infection surrounding terminal airways were still present in 50 and 250 mg/kg/day T-705-treated mice (data not shown).

The 10 day regimens resulted in a more marked dosage-related reduction in virus spread in BALB/c mouse lungs. At 10 dpi, the extent of infection was greatly reduced in 50 mg/kg/day-treated mice, while infection was restricted to very small isolated foci in 250 mg/kg/day-treated mice (Figure 4n and o). However, 10 mg/kg/day did not appear to reduce the severity or extent of infection (Figure S2). At 15 dpi, areas with active infection were further reduced in all groups, including the untreated controls. Relatively small, isolated foci of active infection remained in the 50 mg/kg/day lungs whereas only a few virus-positive cells were detected in 250 mg/kg/day-treated mice (Figure 4p–r and Figure S2). At 45 dpi, some pneumocytes remained positive for virus antigen in 50 or 250 mg/kg/day-treated mice (data not shown), although lung titres were not readily detected (Figure 3d). The antigen distribution around terminal airways in these lungs was also consistent with aerosol reinfection, similar to what was observed with 5 day treatments (data not shown). Together, these findings indicate that a 10 day regimen of T-705 at 250 mg/kg/day essentially prevented the spread of virus infection in the lungs of immunocompromised mice. However, persistent virus replication in the URT likely contributed to initiating pulmonary re-infection.

Emergence of drug-resistant variants

Prolonged viral shedding and antiviral treatment among influenza virus-infected immunocompromised hosts are predisposing factors for the emergence of drug-resistant variants.^{24–31} To investigate whether drug-resistant viruses emerged under T-705 pressure, we selected 23 paired turbinate and lung tissue samples from mice treated for 5 or 10 days. Using cell-based viability assays, we tested recovered viruses for changes in the EC_{50} of T-705 for protecting MDCK cells from a standard infection dose,²³ focusing on timepoints after drug treatment (10 dpi for 5 day and 20 dpi for 10 day treatments) at which there were initial increases in virus titres (Figure 3). The T-705 susceptibility of viruses recovered from the turbinates and lungs of control mice was 36.8–38.1 and 39.7–54.9 μ M, respectively (Table 1). On average, we detected only moderate increases (0.7- to 1.5-fold) in EC_{50} values for viruses

obtained from T-705-treated mice, as compared with controls (Table 1). Thus, phenotypic assays suggest that neither 5 nor 10 day T-705 regimens resulted in viruses with significantly reduced drug susceptibility.

Discussion

T-705 is one of the first influenza virus polymerase inhibitors to achieve market approval (Japan) or enter advanced clinical testing (in Europe and the USA).^{16–18,32} Although adverse side effects in clinical trials have never been reported,³³ teratogenicity, embryotoxicity and mouse toxicity (MLD_{50} , at 707 mg/kg/day) were previously noted in animal models.^{34,35} The antiviral activity of T-705 has been demonstrated for various influenza A viruses, including the highly pathogenic avian influenza (HPAI) A(H5N1)^{16,36} and the emerging human pathogen A(H7N9).³⁷ Here, we demonstrate that T-705 treatment provides survival benefits and suppresses influenza B virus replication in the lungs of immunocompetent and immunocompromised mice.

In BALB/c mice, oral administration of T-705 at ≥ 30 mg/kg/day for 5 days reduced viral titres and improved survival after lethal challenges with influenza A viruses.^{16,36,37} Against HPAI A(H5N1), therapeutic efficacy was retained even when treatments were delayed to 48–96 hpi.^{16,36} When initiated at 24 hpi, our 5 day T-705 regimen required a dosage of ≥ 50 mg/kg/day to significantly increase the survival of influenza B virus-infected BALB/c mice. A dosage of 250 mg/kg/day efficiently cleared BR/08 from the URT and LRT of mice and prevented lung consolidation. Oseltamivir or peramivir therapy also protect against BR/08;^{14,15} however, T-705 promoted earlier virus clearance from the lungs of BALB/c mice, thereby demonstrating better antiviral efficacy than that of NAIs against influenza B virus infection in immunocompetent animals.

A recent study in immunocompromised nude mice has shown that 28 day T-705 monotherapies (20–30 mg/kg once daily) against a mouse-adapted A/California/04/2009 (H1N1)pdm09 virus prolonged survival rate, but did not decrease lung titres, and animals died after completion of treatments.³⁸ In contrast, our regimens with 50 and 250 mg/kg/day lowered lung virus titres in BALB/c mice, suggesting that ≥ 50 mg/kg/day is needed to regulate pulmonary influenza B virus replication. Additionally, prolonged administration with higher dosages of T-705 markedly reduced virus replication and limited lung tissue damage and virus spread. A decrease in BR/08 virus replication in the lungs of immunocompromised mice was not achieved with NAIs,^{14,15} even when oseltamivir was administered for 16 days.¹⁴ A potential limitation of the current study is the number of mice in the compared groups ($n = 3$). Regardless, our findings in murine models collectively demonstrate the potential therapeutic advantage of T-705 against severe influenza B virus infection in immunocompetent and, more importantly, immunocompromised mice. With a different viral protein target, it may also serve as a drug supplement for NAIs in patients infected with drug-resistant viruses. While the

Technologies, Vista, CA, USA). The percentage of each lung field with infection/lesions was calculated using the Aperio ImageScope software. The green lines demarcate the total lung areas measured, red-shaded areas are bronchioles/alveoli with active virus infection (i.e. containing antigen-positive epithelial cells) and yellow lines outline areas with lesions, but negligible antigen (inactive infection). The average percentage of the total lesion area (active and inactive infection) is indicated in black text; active and inactive infections are indicated in red and blue text, respectively. * $P < 0.05$ relative to control group and + $P < 0.05$ relative to 50 mg/kg/day group, as determined by two-way ANOVA with Tukey's multiple comparison post-test.

absence of variant viruses with significantly reduced susceptibility to T-705 supports a high barrier to antiviral resistance as previously observed,^{37,38} continuous monitoring remains a necessity due to the persistent virus replication in immunocompromised hosts. In future studies, it will be important to determine whether delayed and/or combination therapy with T-705 and NAIs would extend the therapeutic window and suppress viral rebound. The recent approval of the endonuclease inhibitor baloxavir marboxil for treatment of influenza also opens new avenues to explore.³⁹

The major difference in T-705 efficacy was observed in its control of BR/08 replication in the URT and LRT of immunocompromised mice. Virus replication restarted in the lungs of all BALB *scid* mice after completion of T-705 administration, whereas nasal titres were essentially unchanged throughout the experiments. Lung viral rebound did not, however, correlate with worsening morbidity and survival. Although no lung titres were obtained at 45 dpi for mice that underwent prolonged high-dosage treatments, viral antigen was still detectable, indicating intermittent alveolar re-infection with limited replication. Similar virus persistence in the URT was noted in transiently immunosuppressed and immunocompromised mice with NAI treatments.^{14,15} Taken together, these results suggest that antiviral compounds are less efficacious in the URT than in the lungs, possibly due to inefficient distribution of the drug to the nasal epithelium, which may be partly explained by differential tissue expression of enzymes required for drug metabolism.⁴⁰

Regulation of virus replication in the URT by resident memory CD8+ T cells is key to limiting viral spread to the LRT and the associated pathology.⁴¹ Because BALB *scid* mice lack mature B and T cells,⁴² virus persistence in the URT probably contributed to the infection dynamics in the LRT. This might also help explain why lung viral titres were unchanged in T cell-deficient nude mice.³⁶ Although antiviral treatment inhibits replication in the LRT, the absence of an efficient mechanism facilitating virus clearance, including in the URT, could result in virus rebound once treatment is completed. Lung infection and impairment in rodents have typically been used as parameters for assessing therapies to prevent or treat influenza-associated disease. However, our efficacy studies,^{14,15} including the current one, show that virus replication in the URT needs to be addressed to minimize the risk of virus relapse in the LRT and, hence, chronic infections in immunocompromised hosts. Thus, it is prudent to include the URT when assessing and interpreting treatment efficacies in antiviral studies.

In conclusion, we demonstrate the therapeutic value of T-705 in controlling influenza B virus infection in immunocompromised mice. Particularly, the combined effect of higher dosage (≥ 50 mg/kg/day) and longer duration (>5 days) of T-705 treatment yields better outcomes. Thus, these are important factors to consider to effectively reduce influenza-associated disease burden and prevent further complications in hosts with defective immune response mechanisms. Overall, our preclinical study indicates that T-705 is a potential alternative option in managing severe influenza B virus infection in high-risk patients.

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Transparency declarations

None to declare.

Supplementary data

Figures S1 and S2 and Tables S1 to S5 are available as [Supplementary data](#) at JAC Online.

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