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Letter

# Tilorone confers robust *in vitro* and *in vivo* antiviral effects against severe fever with thrombocytopenia syndrome virus

*Dear Editor*

Severe fever with thrombocytopenia syndrome virus (SFTSV), an emerging pathogen, is a tick-borne bunyavirus belonging to the genus *Bandavirus* in the family *Phenuiviridae* (Kuhn et al., 2020). This pathogen was first identified in China during the heightened surveillance of acute febrile illness in 2009, and has been reported to cause several outbreaks in eastern Asia areas, including China, Japan, and Korea (Yu et al., 2011). Besides, Vietnam has also reported several confirmed SFTS cases (Tran et al., 2019). The mortality rate in hospitalised patients with SFTSV infection is up to 10%–30%. Moreover, SFTSV has been reported to possibly transmitted by the contact of body fluids from person-to-person, and extensive SFTSV contamination was detected in the patient rooms (Kim et al., 2015). These reports suggest that more stringent isolation measures are needed for the prevention of massive SFTSV outbreak.

Currently, there are no approved vaccines or antiviral drugs available against this bunyavirus. Drug repurposing is an especially attractive strategy to discover anti-SFTSV drugs with potential therapeutic effects. As reported, mycophenolate mofetil, methotrexate, clofarabine, and bleomycin may possess an inhibitory effect of SFTSV RNA synthesis (Yamada et al., 2021), and hexachlorophene may impair the entry of SFTSV into host cells (Yuan et al., 2019). The first clinically approved proteasome inhibitor bortezomib can effectively block the SFTSV NSs mediated immune escape in 293T cells (Liu et al., 2019). Amodiaquine, an antimalarial agent, showed promising inhibitory activity against SFTSV, whereas the *in vivo* activity is still unclear (Baba et al., 2017). The broad-spectrum antiviral drugs ribavirin and favipiravir have been reported to be effective against SFTSV *in vitro* and in animal models; however, retrospective clinical studies revealed limited efficacy of ribavirin, as well as favipiravir failed to protect hospital patients with high viral load (Liu et al., 2013; Li et al. 2018, 2021). The anti-hypertensive drugs, benidipine and nifedipine, have been shown to be effective against SFTSV *in vitro* and *in vivo*. Further, a retrospective clinical investigation suggested the therapeutic potential of nifedipine in terms of reducing viral load and improving survival. More approved drugs with improved efficacy and safety are urgently needed (Li et al., 2019).

Here, we report an optimized high-throughput drug repurposing assay based on the viral cytopathological effect (CPE). At first, a CPE-based high-throughput method was established using human-derived Huh7 cells. A drug library containing 2572 approved drugs was screened using a luminescence-based cell viability assay, among which only two drugs showed a cell protection efficiency above 30% at 3 μmol/L, including tilorone dihydrochloride (Fig. 1A).

Tilorone, trade name amixin or lavomax, has been registered in Russian, Ukraine, Kazakhstan and some other countries for antiviral and immunoregulatory activities, and listed in the list of vital and essential medicines of the Russian Federation (Ekins et al., 2020). Also, tilorone has been approved for the treatment of idiopathic pulmonary fibrosis as an orphan designation by the European Medicines Agency (Vartiainen et al., 2018). To verify the anti-SFTSV activity of tilorone, further dose-dependent CPE assay was performed, and tilorone exhibited prominent antiviral potency with a cell protection rate of 76.57% at 1 μmol/L, along with a concentration for 50% of maximal effect ($EC_{50}$) of 0.42 ± 0.02 μmol/L and a selection index of approximately 23.81 (Fig. 1B). To further evaluate the antiviral efficacy of tilorone, infectious viral particles and viral RNA were quantified after treatment of the compound, and dose-dependent inhibition of virus and RNA yield was observed. Treatment with 1 μmol/L tilorone led to 99.77% inhibition of infectious viral particle yield compared with the control group, and 65.74% inhibition of cellular viral RNA was observed at 1 μmol/L (Fig. 1C).

To explore whether tilorone exerts its antiviral effect by stimulating host innate immunity, we evaluated the antiviral effect of tilorone in Huh7.5 cells. Huh7.5 cell is Huh7 cell whose RIG-I pathway is knocked out, which may lead to insufficient immune activation of antiviral response, while does not affect the replication of SFTSV RNA (Supplementary Fig. S1) (Binder et al., 2007; Blight et al., 2002). CPE protection activity was not observed in Huh7.5 cells. As shown in Fig. 1D, treatment with 1 μmol/L tilorone failed to protect the CPE of Huh7.5 cells. In addition, production of infectious viral particles and viral genome replication was not effectively inhibited in Huh7.5 cells when treated with the same concentration of tilorone, while an inhibitory effect was observed in Huh7 cells (Fig. 1E). We then performed an immunofluorescence assay to compare the antiviral effect of tilorone in Huh7 and Huh7.5 cells at the viral protein level. The production of viral protein was completely inhibited in Huh7 cells treated with 3 μmol/L tilorone. Nonetheless, only slightly inhibition was observed in Huh7.5 cells after treatment with the same drug concentration (Fig. 1F). The above results suggested that tilorone was highly potent in inhibiting SFTSV in human cells and that this inhibition is associated with innate immunity. Next, to achieve better antiviral efficacy, we combined tilorone with favipiravir and observed enhanced inhibition of SFTSV induced CPE in Huh7 cells (Supplementary Fig. S2). The robust synergistic effect of the two drugs with different mode of action provided an optimized strategy for the clinical management of SFTSV infections, especially for the severe cases.

*In vivo* protection efficacy is a key parameter to assess the antiviral

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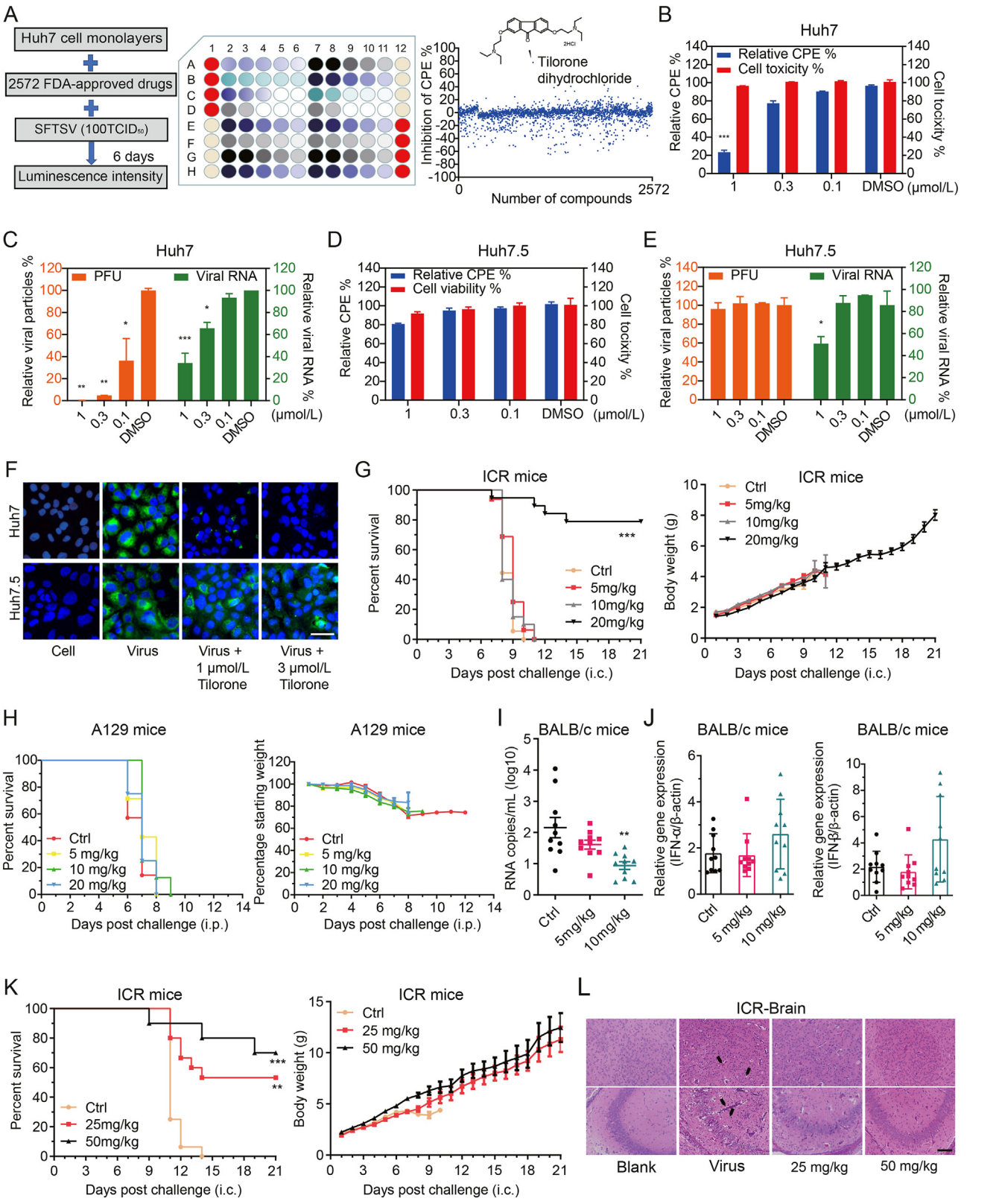


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potential of the antiviral agents. To evaluate the *in vivo* antiviral efficacy of tilorone against SFTSV, we first established a lethal virus challenge model using wild-type 1-day-old suckling ICR mice as previous described with some modifications (Supplementary Fig. S3A) (Ning et al., 2019). After challenge with $5 \times 10^3$ plaque forming units (PFU) of virus intracranially, the mice were administered tilorone intraperitoneally once daily at 1–7 days post challenge. The administration of 20 mg/kg of tilorone protected 78.94% of the mice from lethal challenge, compared with none in the vehicle group at 21 days post infection (Fig. 1G). To explore whether tilorone exerts *in vivo* antiviral efficacy through stimulation of host innate immunity, we repeated the therapeutic assay in the *Ifna*$^{-/-}$ mice A129, whose type-I interferon (IFN) receptors were knocked out (Li et al., 2013; Yang et al., 2020). Intraperitoneal challenge with 10 PFU of virus led to a fatality rate of 100% in the 6- to 8-week-old female A129 mice model within 8 days. Although less virus was needed for 100% fatality in adult A129 mice than in suckling ICR mice, the same drug regimen failed to offer any protection to A129 mice (Fig. 1H, Supplementary Fig. S3B). The fact that tilorone failed to protect from lethal challenge or prolong survival in type-I IFN pathway-deficient mice suggested that the IFN pathway played a role in the *in vivo* antiviral process.

To further address this issue, we developed a viremia model based on wild-type 6-week-old female BALB/c mice. Tilorone was administered 12 h prior to challenge, at the time point of challenge, and 12 h after challenge with $5 \times 10^5$ PFU of SFTSV intraperitoneally, and whole blood was collected at 24 h post challenge. Viremia and IFN levels were quantified using RT-PCR. The results revealed that viremia was dose-dependently inhibited by more than 10 folds after treatment with 10 mg/kg tilorone (Fig. 1I). A general increase in IFN-α and IFN-β was also observed, suggesting the potential activation of the innate immune process after drug administration (Fig. 1J). Besides, inflammation related expression of TNF-α and IL-10 was significantly reduced after administration of tilorone, indicating the release of SFTSV infection induced inflammatory response (Supplementary Fig. S4). The above results further suggested that tilorone exerted an *in vivo* protective effect against SFTSV challenge through the regulation of the innated immunity.

As an immune stimulator, we hypothesised that tilorone may also have a prophylactic effect against SFTSV infection. To address this question, we pre-treated suckling ICR mice with tilorone for three consecutive days and challenged the mice intracranially with a lethal dose of SFTSV ($5 \times 10^4$ PFU). Notably, we found that pre-treatment with 50 mg/kg of tilorone protected 70% of mice from death, and 25 mg/kg of tilorone protected 53.33% of mice, while all mice in the control group died within 14 days (Fig. 1K). To verify the prophylactic effect, the mice were sacrificed on day 6 post challenge for pathological analysis. In the vehicle group, direct virus infection of the brain led to extensive vacuolation degeneration of nerve cells and brain congestion, while no tissue damage was observed in the brains of mice pre-treated with tilorone (Fig. 1L). We observed that intraperitoneal administration of tilorone led to protection against intracranial viral challenge, which suggested that extensive stimulation of innate immunity in several organs caused by tilorone might become a multi-functional antiviral strategy.

In summary, our results demonstrated that tilorone, an approved drug, was very potent in treating SFTSV infection and was highly effective in stimulating multiorgan innate immunity to combat viral infection. Considering that tilorone is effective against other viruses including SARS-CoV-2 (Xiao et al., 2020), we are optimistic that this drug may have the potential to become a "universal vaccine" against viral pandemics.

## Footnotes

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2022.01.014.

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