Virologica Sinica 39 (2024) 802–811

Contents lists available at ScienceDirect

Virologica Sinica

journal homepage: www.keaipublishing.com/en/journals/virologica-sinica
www.virosin.org

Research Article

Antiviral activity of vitamin D derivatives against severe fever with thrombocytopenia syndrome virus *in vitro* and *in vivo*

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ARTICLE INFO

Keywords:
Severe fever with thrombocytopenia syndrome virus (SFTSV)
Antiviral activity
Vitamin D derivatives
Drug combination

ABSTRACT

Severe fever with thrombocytopenia syndrome virus (SFTSV) is a tick-borne virus that causes the severe fever thrombocytopenia syndrome, which manifests as fever and haemorrhage, accompanied by severe neurological complications. To date, no specific antiviral drugs have been approved for this indication. Herein, we investigated whether vitamin D derivatives inhibit SFTSV both *in vitro* and *in vivo*. An *in vitro* study demonstrated that vitamin D derivatives significantly suppressed viral RNA replication, plaque formation, and protein expression in a dose-dependent manner. Subsequently, *in vivo* studies revealed that doxercalciferol and alfacalcidol were associated with increased survival and reduced viral RNA load in the blood. Time-of-addition assay suggested that vitamin D derivatives primarily acted during the post-entry phase of SFTSV infection. However, cytopathic effect protective activity was not observed in RIG-I immunodeficient cell line Huh7.5, and the administration of vitamin D derivatives did not improve the survival rates or reduce the blood viral loads in adult A129 mice. Further transcriptome exploration into the antiviral mechanism revealed that alfacalcidol stimulates host innate immunity to exert antiviral effects. To expand the application of vitamin D derivatives, *in vitro* and *in vivo* drug combination assays were performed, which highlighted the synergistic effects of vitamin D derivatives and T-705 on SFTSV. The combination of alfacalcidol and T-705 significantly enhanced the therapeutic effects in mice. This study highlights the potential of vitamin D derivatives against SFTSV and suggests that they may have synergistic effects with other compounds used in the treatment of SFTSV infection.

1. Introduction

Severe fever with thrombocytopenia syndrome virus (SFTSV) is a novel bunyavirus, which belongins to *Bandavirus* genus, *Phenuiviridae* family, *Bunyavirales* order (Kuhn et al., 2020). SFTSV was first described in China and was thereafter reported in Korea and Japan (Kim et al., 2013; Takahashi et al., 2014; Yu et al., 2011). SFTSV infection is characterised by fever and haemorrhage and is associated with severe neurological complications, with the mean mortality rate of 7.3% (Liu et al., 2014). Previous studies have identified nucleoside analogues, including T-705 and ribavirin, as inhibitors with beneficial anti-SFTSV activity both *in vitro* and *in vivo* (Li et al., 2021; Shimojima et al., 2014; Tani et al., 2016). However, the therapeutic benefits of ribavirin remain controversial, and ribavirin treatment does not affect the viral load in patients (Liu et al., 2013; Lu et al., 2015). Although the administration of T-705 may reduce morbidity and mortality in patients infected with SFTSV, the potential of this compound as a treatment for SFTSV infection remains to be explored, including the optimal timing, dosage, and duration of treatment (Li et al., 2021). Considering that SFTSV is widespread worldwide, and associated infectious disease outbreaks may reach pandemic proportions, novel treatments are urgently required to combat SFTSV infections.

SFTSV has a spherical shape with a diameter of 80–100 nm (Yu et al., 2011). SFTSV genome is a segmented, single-stranded RNA. Viral genomes are composed of three distinct genes: large (*L*), medium (*M*), and small (*S*). The *L* gene encodes an RNA-dependent RNA

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https://doi.org/10.1016/j.virs.2024.08.007
Received 19 April 2024; Accepted 15 August 2024
Available online 20 August 2024

polymerase that functions as a viral transcriptase and replicase. The *M* gene encodes the viral surface glycoproteins, which consist of two glycoproteins, Gn and Gc. These proteins form the viral envelope. The *S* gene encodes the nuclear protein (NP) and the non-structural protein (NSs). The NP protein plays a pivotal role in the encapsulation of viral RNA, while the NSs protein impedes the production of host interferons (Bopp et al., 2020; Kim et al., 2023). The viral replication cycle comprises a series of interconnected processes, including adsorption, entry, replication, transcription, translation, and assembly. Compounds with antiviral properties against SFTSV affect the viral replication cycle.

Vitamin D is a fat-soluble molecule that plays an important role in calcium and phosphorus homeostasis. Vitamin D and its derivatives have various pharmacological effects, influencing calcium and phosphorus metabolism, bone growth, immune function, and serving as antiviral therapy (Ismailova et al., 2021; Janoušek et al., 2022). Vitamin D was initially developed to treat rickets, a condition that causes bone softening in children. Currently, vitamin D products are widely used to treat osteoporosis (Christakos et al., 2019; Janoušek et al., 2022). Vitamin D is metabolically activated by the liver and kidneys before it becomes biologically active, while vitamin D derivatives do not require metabolic activation. Furthermore, vitamin D derivatives exhibit a relatively high affinity for vitamin D binding proteins and a prolonged half-life. As reported, several vitamin D derivatives have shown efficacy in hyperproliferative diseases, immune dysfunction, and endocrine and metabolic bone diseases. For instance, doxercalciferol has been found to reduce the elevated parathyroid hormone levels. Alfacalcidol has been approved for the treatment of osteoporosis. Calcipotriol is used to treat psoriasis (Brown, 2001; Chen et al., 2020; Kubodera, 2009). Previous studies have reported that vitamin D has antiviral activity against Coxsackie virus, Zika virus, dengue virus, and other viruses (Fernandez et al., 2023; Jaratsittisin et al., 2020; Qu et al., 2017). In addition, adding active vitamin D to the standard treatment may benefit patients with COVID-19 pneumonia (Ali, 2020). The antiviral activity of vitamin D derivatives is receiving increasing attention.

Here, to address the immediate need for the identification and development of antivirals against SFTSV, this study aimed to elucidate the inhibitory activity of vitamin D derivatives against SFTSV both *in vitro* and *in vivo*, and preliminarily explore their potential antiviral mechanisms. Moreover, we hope to provide evidence that vitamin D derivatives used in different drug combinations are promising candidates for the treatment of SFTSV infection.

2. Materials and methods

2.1. Cells, viruses, and animals

Huh7 cells and BHK cells were obtained from the National Infrastructure of Cell Line Resource, China. MDCK cells and Vero cells were purchased from the American Type Culture Collection. Huh7.5 cell line was a gift from Prof. Charles M. Rice. All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin at 37 °C and 5% CO_2.

The SFTSV strains utilized in this study were procured from Ditan Hospital of Capital Medical University. Researchers in our laboratory isolated SFTSV strains from clinical samples and amplified and titrated the virus in Huh7 cells. Influenza strains ZX1109 were preserved in the laboratory. DENV (serotype 2, New Guinea C strain, GenBank M29095) and Ross River virus (ATCC, cat. No. VR-3345) were purchased from the American Type Culture Collection.

Six-week-old BALB/c mice and one-day-old ICR suckling mice were purchased from Beijing Vital River Laboratory Animal Technology, China. A129 mice (*Ifnar*$^{-/-}$) were housed in an environmentally controlled, specific pathogen-free (SPF) animal facility at the Beijing Institute of Pharmacology and Toxicology.

2.2. Compounds

Vitamin D2, Doxercalciferol, 5,6-trans-Vitamin D2, Ercalcidiol, 1α,25-Dihydroxypre vitamin D2, 24,25-Dihydroxypre vitamin D2, Seocalcitol, Calcifediol, Vitamin D3, Alfacalcidol, Tacalcitol, 7-Dehydrocholesterol, Secalciferol, Ergosterol, Paricalcitol, Calcipotriol, 3-*O*-(2-Aminoethyl)-25-hydroxyvitamin D3, Maxacalcitol, Inecalcitol, Eldecalcitol, TEI-9647, TEI-9648, Vitamin D4, 1-alpha-hydroxy VD4, T-705, Oseltamivir and NITD008 were purchased from MedChemExpress. Compounds were dissolved in DMSO upon receipt and stored at −20 °C.

2.3. Cytopathic effect (CPE) inhibition assay and cytotoxicity assay

CPE inhibition and cytotoxicity assays were conducted using different cell lines as previously described, respectively (Yan et al., 2022; Yang et al., 2022; Zhao et al., 2022). Vitamin D and derivatives were added to 96-well plates in a three-fold dilution series, with concentrations ranging from 100 μmol/L to 0.005 μmol/L. Subsequently, indicated virus was inoculated into the cells at 100 $TCID_{50}$ (50% tissue culture infectious dose). T-705, Oseltamivir and NITD008 were employed as positive controls. Cell viability was measured by CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega, China) following the manufacturer's instructions.

2.4. Viral yield reduction assay

Huh7 cells were seeded in 12-well plates at a density of 2.0×10^5 cells/well and cultured overnight. The cells were then infected with SFTSV at a multiplicity of infection (MOI) of 0.01. The test compounds were administered at varying concentrations. After 48 h of incubation, Total RNA was extracted from the cell samples using TRIzol reagent (Invitrogen) and quantitative real-time polymerase chain reaction (qRT-PCR) using the One Step PrimeScript™ RT-PCR Kit (TaKaRa, Japan), as previously described (Baba et al., 2023; Li et al., 2022). The levels of SFTSV RNA were quantified. The specific primers and probes utilized for SFTSV were as follows: forward primer, 5′-GGGTCCCTGAAGGAGTTGTAAA-3′; reverse primer, 5′-TGCCTTCACCAAGACTATCAATGT-3′; and probe, 5′-FAM-TTCTGTCTTGCTGGCTCCGCGC-TAMRA-3′. The number of viral RNA copies was determined based on the cycle threshold (CT) for each sample, employing a standard curve of known copy numbers. The supernatant was collected for quantification of infectious viral particles using a plaque formation assay.

2.5. Plaque reduction assay

Huh7 cells were seeded in 12-well plates and cultured overnight, after which they were infected with SFTSV at an MOI of 0.01. The test compounds were administered at varying concentrations. After 48 h, supernatants were collected. The supernatant was then 10-fold diluted and inoculated into cells, which were incubated for 2 h. The cells were then washed three times with PBS. Subsequently, a 2% low-melting-point agarose solution in DMEM was added to the cell monolayer. Upon the appearance of plaques three days later, the cells were fixed with 4% formaldehyde and stained permanently with 1% crystal violet.

2.6. Immunofluorescence assay (IFA)

IFA was performed as previously described (Yan et al., 2022). Cells were seeded in 96-well plates at a density of 5×10^3 cells/well and cultured overnight, after which they were infected with SFTSV at an MOI of 0.01. The test compounds were administered at varying concentrations. After 72 h, cells were fixed with 4% paraformaldehyde and 0.1% Triton X-100, and then blocked with 5% bovine serum albumin (BSA). The cells were then stained with a primary antibody (Abnova), followed by staining with goat anti-rabbit IgG (H + L) Alexa Fluor TM Plus 546 (Invitrogen). The nuclei were stained with a fluorescent solution of

Hoechst 33342 (Invitrogen), and images of the cells were captured using a Leica DMi8 inverted microscope (Leica DMi8®).

2.7. In vivo antiviral efficacy

For mouse lethal protection experiments, one-day-old ICR suckling mice were injected intraperitoneally (i.p.) with 1×10^5 plaque forming unit (PFU)/mouse. At 24 h post-infection (h.p.i.), the mice were i.p. administered doxercalciferol (0.3 or 0.15 μg/kg), alfacalcidol (0.6 or 0.3 μg/kg), or PBS. The treatment was continued daily for 8 days. Mice were monitored daily for weight and mortality until 21 days. The six-week-old BALB/c mice were i.p. injected with SFTSV (1×10^5 PFU/mouse). Doxercalciferol (30 μg/kg, 15 μg/kg) and alfacalcidol (30 μg/kg, 15 μg/kg) were administered orally before, during, and at 12 h intervals after infection. At 24 h.p.i., viral loads were determined by qRT-PCR.

For the lethal infection model of A129 mice, six to eight-week-old A129 mice (*Ifnar*$^{-/-}$) were i.p. injected with SFTSV (10 PFU/mouse). Meanwhile, mice in different groups were i.p. administrated with different doses of doxercalciferol (10 μg/kg, 5 μg/kg), alfacalcidol (10 μg/kg, 5 μg/kg), and PBS. The survival and weight of A129 mice (*Ifnar*$^{-/-}$) were monitored daily until 8 days. Doxercalciferol (10 μg/kg, 5 μg/kg) and alfacalcidol (10 μg/kg, 5 μg/kg) were administered every 12 h for three doses. The blood of A129 mice (*Ifnar*$^{-/-}$) was collected at 24 h.p.i., viral load in blood was quantified by qRT-PCR.

2.8. Time-of-addition assay

The time-of-addition assay was performed as previously described with some modifications (Yan et al., 2022). Huh7 cells were seeded in 12-well plates at a density of 2.0×10^5 cells per well, and subsequently infected with SFTSV at an MOI of 0.01. T-705 (100 μmol/L) was utilized as a positive control. At 24 h.p.i., cells were harvested, total RNA was extracted, and the assay was subjected to quantitative reverse transcription polymerase chain reaction (qRT-PCR).

2.9. Combinational effects of vitamin D derivatives and T-705 in vitro and in vivo

A combination of vitamin D derivatives and T-705 was performed as described previously, with some modifications (Gao et al., 2021; Ma et al., 2018). Huh7 cells were seeded in 96-well plates at a density of 5×10^3 cells/well and cultured overnight, followed by infection with 100 $TCID_{50}$ SFTSV. Subsequently, the addition of vitamin D derivatives and T-705 in different dilutions (for doxercalciferol, 0–6.67 μmol/L; for alfacalcidol or 1-alpha-hydroxy VD4, 0–2.24 μmol/L, T-705 0–166.67 μmol/L). For cotreatment, two single compound controls were present on each plate to obtain valid results. After incubation at 37 °C for 6 days until evidence of CPE, the antiviral effect was measured using CellTiter-Glo cell viability assay. Data were analyzed using the MacSynergy II software (Prichard and Shipman, 1990).

The *in vivo* antiviral activity of the combination of alfacalcidol with T-705 using an infectious lethal model in one-day-old ICR suckling mice. The mice were randomly divided into four groups: one viral control group, one monotherapy group T-705 (25 mg/kg), and two groups treated with the combination: the combination of alfacalcidol (1.2 μg/kg) with T-705 (25 mg/kg) and the combination of alfacalcidol (0.6 μg/kg) with T-705 (25 mg/kg). T-705 was formulated as a sterile suspension of 0.5% carboxymethylcellulose sodium. The treatment was continued daily for 8 days.

2.10. Transcriptome analysis

Huh7 cells were seeded in 12-well plates at 1×10^5 cells/well, followed by infection with SFTSV at an MOI of 0.01. Huh7 cells were incubated with alfacalcidol or DMSO for 24 h at 37 °C. Total RNA was extracted using the TRIzol Reagent according to the manufacturer's instructions. A Bioanalyzer (NanoDrop, Thermo Fisher) was used to assess RNA quality. Only high-quality RNA samples (OD260/280 = 1.8–2.0; OD260/230 = 2.0–2.2) were used to build the sequencing library. The samples were sequenced using an Illumina NovaSeq 6000 supplied by Shanghai Majorbio Bio-Pharm Technology Co., Ltd.

2.11. Statistical analyses

Statistical analyses were performed using GraphPad Prism 9 software. The means ± standard deviations of the data collected from three independent experiments were presented. Data were analyzed using an unpaired two-tailed Student's *t*-test or one-way ANOVA. Survival curves were analyzed using the log-rank test. Statistical significance was set at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

The combined effects were assessed using MacSynergy II. The 3-dimensional peaks above and below the plane represented quantitative synergy and antagonism of the interaction of the indicated drugs, respectively.

3. Results

3.1. Vitamin D derivatives exert antiviral effects against SFTSV infection in vitro

In previous work, our laboratory conducted *in vitro* high-throughput screening and identified two vitamin D derivatives among 2572 commercially available compounds with good inhibitory effects against SFTSV infection (Yang et al., 2022). Based on these findings, we purchased 25 vitamin D and derivatives to screen for their anti-SFTSV activity (Supplementary Table S1 and Fig. S1). Among these derivatives, three were selected for further investigation in Huh7 cells: doxercalciferol, alfacalcitol, and 1-alpha-hydroxy VD4 (Fig. 1A), which are derivatives of vitamins D2, D3 and D4, respectively. To assess the cytotoxicity of vitamin D derivatives, a concentration gradient was employed for cell treatment, and cell viability was assayed using the CellTiter-Glo cell viability assay kit. The results showed non-cytotoxic concentrations of 9.00 μmol/L, 5.00 μmol/L, and 9.00 μmol/L, for doxercalciferol, alfacalcidol, or 1-alpha-hydroxy VD4, respectively. A cytopathic effect (CPE) inhibition test was performed to assess the effects of vitamin D derivatives on SFTSV-induced CPE. The results showed that the half-maximal effective concentration (EC_{50}) values were 1.98 ± 1.30 μmol/L, 1.59 ± 0.35 μmol/L, and 2.72 ± 0.40 μmol/L, for doxercalciferol, alfacalcidol, or 1-alpha-hydroxy VD4, respectively (Fig. 1B–D). T-705 was used as the positive control and the EC_{50} level was 11.97 ± 4.94 μmol/L (Fig. 1E). The results demonstrated that both the vitamin D derivatives and T-705 exhibited dose-dependent protection against CPE in SFTSV-infected Huh7 cells, indicating their stable CPE inhibitory effects.

3.2. Vitamin D derivatives decrease viral RNA, infectious viral particles, and viral protein levels in SFTSV-infected cells

To further characterize the potential of vitamin D derivatives against SFTSV, a virus yield reduction assay was conducted. The inhibitory effects of three vitamin D derivatives on viral RNA replication and infectious viral particle production were evaluated. The results showed that doxercalciferol, alfacalcidol, and 1-alpha-hydroxy VD4 inhibited viral RNA replication (Fig. 2A) and the propagation of infectious virus particles in a dose-dependent manner (Fig. 2B). These findings indicate that vitamin D derivatives can impede viral replication and the infectivity of viral particles. The level of viral protein expression is one of the most important indicators of antiviral efficacy of compounds. We performed immunofluorescence assay (IFA) to evaluate the inhibitory effects of the three vitamin D derivatives on viral protein levels. The results showed that vitamin D derivatives decreased the inhibition of SFTSV protein

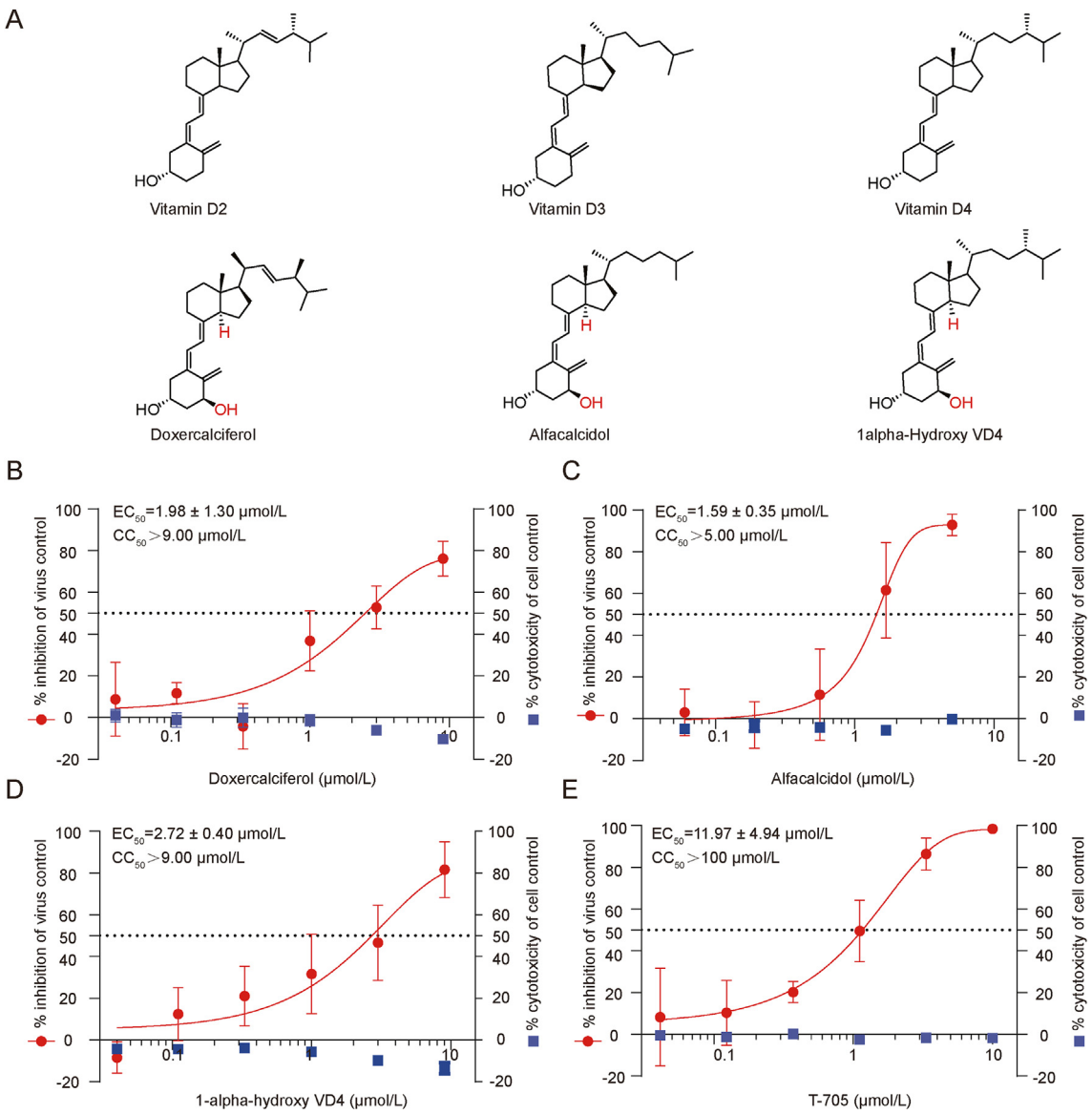

Fig. 1. Antiviral effects of vitamin D derivatives and T-705 *in vitro*. **A** Structural formula of vitamin D and derivatives. **B-E** Huh7 cells were treated with different doses of the indicated antiviral agents for 6 days after infection with 100 $TCID_{50}$ SFTSV. The left Y-axis and right Y-axis represent the percentage inhibition of virus control and cytotoxicity of cell control, respectively. The inhibition rate and cell viability of antiviral compounds against cytopathic effect (CPE) resulting from SFTSV infection are depicted in red and blue, respectively. The experiments were performed in triplicates. Half-maximal effective concentration (EC_{50}) and half-maximal cytotoxic concentration (CC_{50}) were calculated using parametric non-linear regression models and plotted using GraphPad Prism 9.

expression (Fig. 2C and Supplementary Fig. S2). In summary, vitamin D derivatives exhibit anti-SFTSV activity in *vitro*.

3.3. Vitamin D derivatives exhibit anti-SFTSV activity in vivo

Based on *in vitro* antiviral assay results, doxercalciferol and alfacalcidol were selected for further *in vivo* antiviral evaluation. We evaluated the therapeutic effects of doxercalciferol and alfacalcidol on SFTSV infection in one-day-old ICR mice, a lethal challenge model of SFTSV infection. The results revealed that both doxercalciferol and alfacalcidol enhanced the survival rates of SFTSV-infected one-day-old ICR mice. Monotherapy with doxercalciferol (0.3 μg/kg/d) and alfacalcidol (0.6 μg/kg/d) provided 40% and 64% protection against lethal SFTSV infections, respectively (Fig. 3A). The body weights of the one-day-old suckling mice in the doxercalciferol and alfacalcidol treated groups increased steadily throughout the experiment. Moreover, changes in the viremia after doxercalciferol or alfacalcidol administration (15 and 30 μg/kg, every 12 h for 3 days) were evaluated in SFTSV-infected six-week-old BALB/c mice. The results showed that SFTSV RNA levels in the doxercalciferol- and alfacalcidol-treated groups were significantly lower than those in the control group (Fig. 3B). In addition, the administration of high doses of doxercalciferol or alfacalcidol to six-week-old BALB/c mice did not result in death and led to a consistent increase in body weight (Supplementary Fig. S3). In summary, treatment with the vitamin D derivatives partially inhibited SFTSV infection and prolonged the survival of infected mice.

3.4. Vitamin D derivatives act primarily in the post-entry phase of the SFTSV infection

To determine the mode of action of vitamin D derivatives, we performed a time-of-addition experiment. Vitamin D derivatives were added

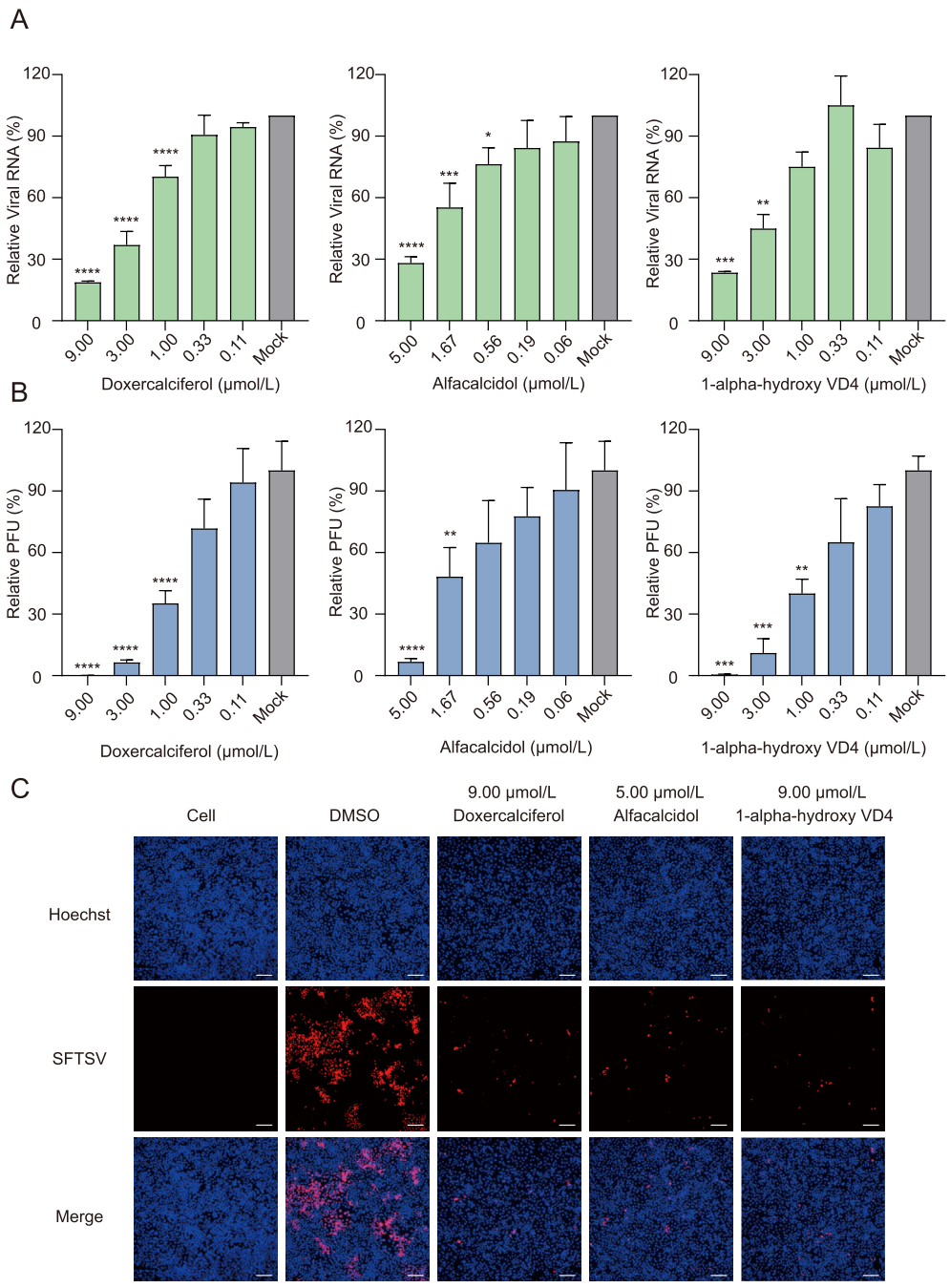

Fig. 2. Viral RNA, viral particle, and viral protein yield reduction assays. **A** Huh7 cells were inoculated with SFTSV at a multiplicity of infection (MOI) = 0.01 in the presence of doxercalciferol, alfacalcidol, or 1-alpha-hydroxy VD4. After 48 h, total RNA was extracted, and qRT-PCR analysis was performed to measure the viral RNA expression. **B** The infectious viral particles in the supernatant of SFTSV virus treated with various concentrations of doxercalciferol, alfacalcidol, or 1-alpha-hydroxy VD4 were quantified using a plaque assay. **C** Immunofluorescence assay (IFA) of SFTSV protein in SFTSV-infected Huh7 cells treated with vitamin D derivatives. Huh7 cells were treated with different concentrations of compounds and infected with SFTSV at an MOI of 0.01. Cells were fixed for an immunofluorescence assay at 72 h post-infection. The viral SFTSV protein (red) was stained with rabbit serum against SFTSV HB29 followed by incubation with secondary antibodies, namely Alexa 546-labeled goat anti-rabbit, and cell nuclei were stained using Hoechst 33,342 (blue). Scale bar = 100 μm. One-way ANOVA was used for statistical analysis. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

to treat SFTSV-infected Huh7 cells at different stages of viral infection, including the adsorption, entry, replication, and release phases. The overall replication efficiency of the viruses was determined using qRT-PCR. The results revealed that the three vitamin D derivatives inhibited SFTSV RNA production at stages III and IV (Fig. 4A). This suggests that vitamin D derivatives inhibit SFTSV at the post-entry stage of the viral replication cycle.

3.5. Vitamin D derivatives exhibit in vitro and in vivo antiviral activity against anti-SFTSV infection through regulation of innate immunity

Innate immune signaling triggered by virus infection, which in turn modulates retinoic acid-inducible gene I (RIG-I) activity and IFN induction. To determine whether the antiviral action of vitamin D derivatives is contingent on innate immunity, we selected the RIG-I

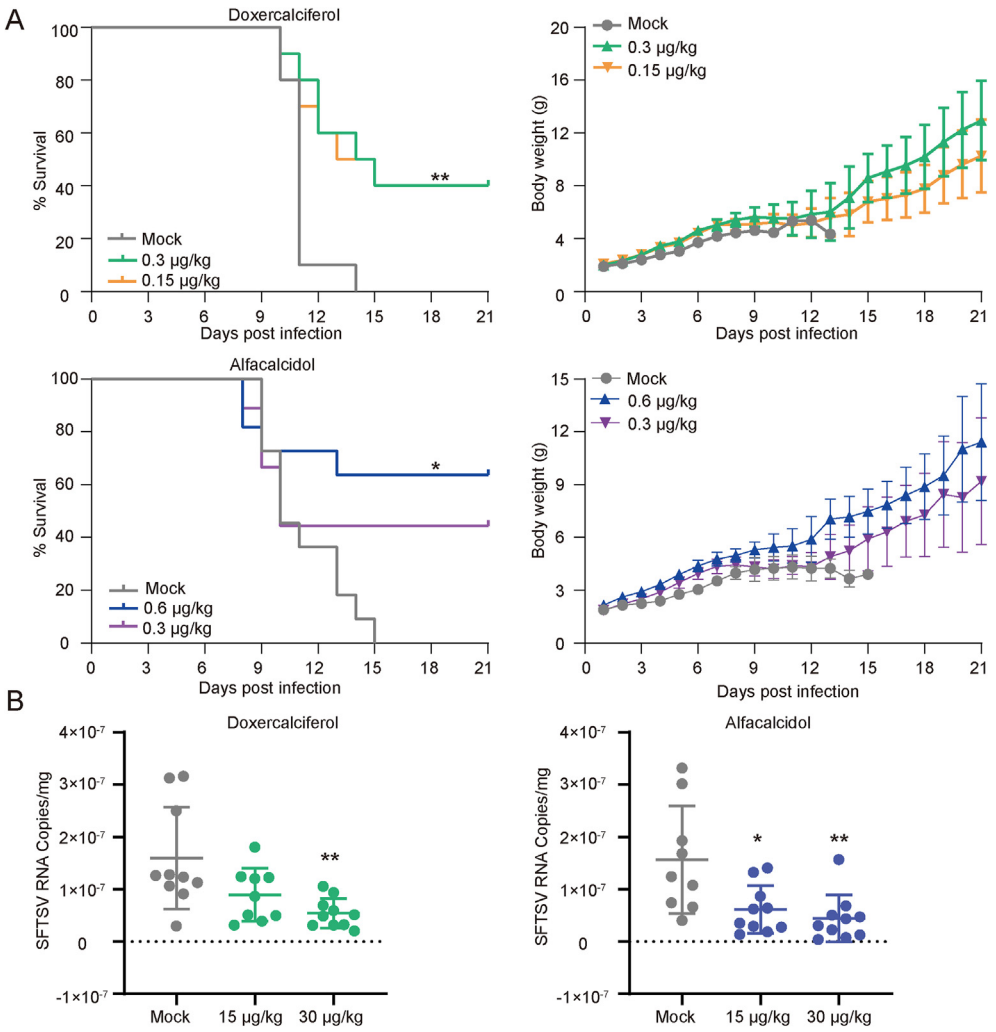

Fig. 3. Effects of doxercalciferol or alfacalcidol on SFTSV infection in one-day-old ICR mice and six-week-old BALB/c mice. **A** Survival curves and body weight curves. Doxercalciferol, alfacalcidol, or PBS (Mock) with the indicated doses were administrated for 8 consecutive days. Mouse weight was recorded daily for 21 days. **B** Viremia changes in six-week-old BALB/c mice. BALB/c mice were i.p. injected with SFTSV (1×10^5 PFU/mouse). Doxercalciferol (15 and 30 μg/kg) or alfacalcidol (15 and 30 μg/kg) were administered orally every 12 h for 3 doses. At the same time of infection, doxercalciferol or alfacalcidol mice were administrated orally for the second time. Viremia was assessed at 24 h post infection, viral loads in the blood were quantified using qRT-PCR and were displayed as viral RNA copy number per millilitre of blood. Survival curve analysis was performed using the log-rank test. Viremia analysis was performed using one-way ANOVA. $^*P < 0.05$, $^{**}P < 0.01$.

immunodeficient cell line Huh7.5 and A129 mice (*Ifnar*$^{-/-}$) for preliminary investigation. The results showed that Huh7.5 cells infected with SFTSV and treated with the same concentration of vitamin D derivatives did not exhibit CPE protective activity (Fig. 5A). T-705 was used as a positive control, with an EC_{50} was 1.52 ± 0.08 μmol/L (Supplementary Fig. S4). Although a lower amount of virus was required to cause lethal infection in adult A129 mice than in suckling ICR and adult BALB/c mice, neither doxercalciferol nor alfacalcidol improved the survival rates or reduced the blood viral load in adult A129 mice (Fig. 5B and C). To further elucidate the potential molecular mechanisms associated with the antiviral effects of the vitamin D derivatives, we analyzed the transcriptome profiles of Huh7 cells infected with SFTSV in the presence and absence of alfacalcidol (Supplementary Fig. S5). Heatmap displayed differentially expressed genes (DEGs) in Huh7 cells, sorted by hierarchical clustering high gene expression is represented in red, while low gene expression is represented in blue (Fig. 5D). The analysis showed significant upregulation of genes related to innate immunity, such as CXCL6 and TNFSF10. These findings may provide new directions for future anti-SFTSV research.

3.6. *The combination of vitamin D derivatives and T-705 synergistically inhibited SFTSV infection in vitro and in vivo*

To address the limited antiviral efficacy of vitamin D derivatives, we investigated the potential enhancement by combining them with other broad-spectrum antivirals. T-705 is a broad-spectrum antiviral agent, which has been clinically explored for the treatment of patients with SFTSV (Furuta et al., 2013; Li et al., 2021). We evaluated the synergistic antiviral effects of vitamin D derivatives in combination with T-705 *in vitro* and *in vivo*. The co-administration of doxercalciferol, alfacalcidol, and 1-alpha-hydroxy VD4 with T-705 in SFTSV-infected cells, along with specified concentrations of the vitamin D derivatives achieved a significant synergistic antiviral effect (Fig. 6A). *In vivo* results showed that T-705 (25 mg/kg/d) provided 66.67% protection as a monotherapy,

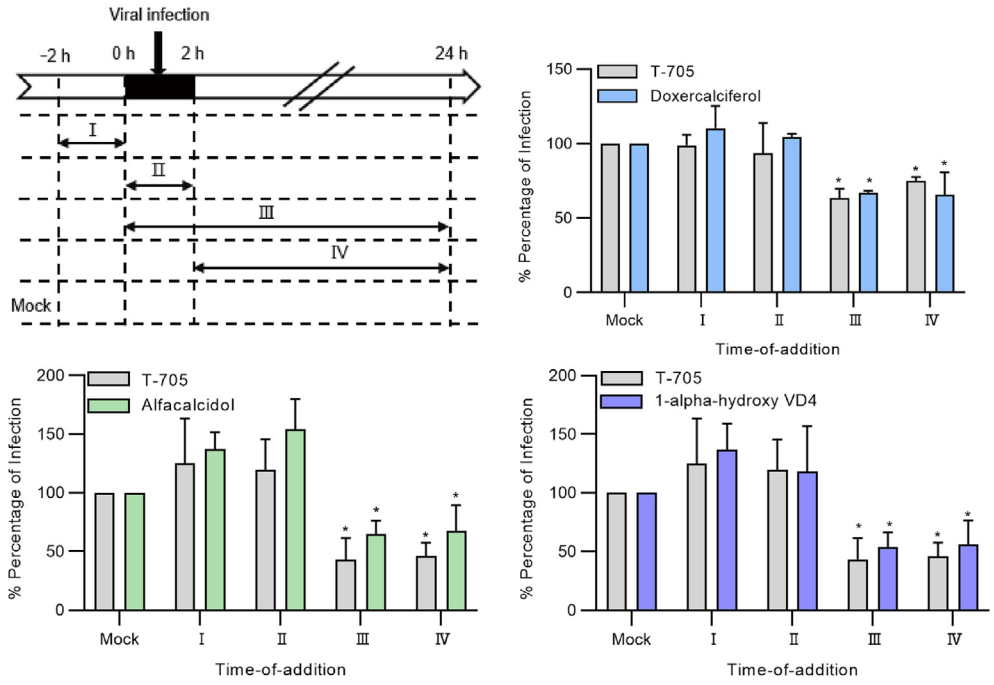

Fig. 4. Time-of-addition assay. At various time points, the addition or removal of vitamin D derivatives was used to treat the SFTSV-infected Huh7 cells, with viral RNA quantified at 24 h using qRT-PCR analysis. Comparisons between vitamin D derivatives group and T-705 group were performed using Student's unpaired *t*-test, $^{*}P < 0.05$.

while the combination of T-705 (25 mg/kg/d) + alfacalcidol (1.2 μg/kg/d) resulted in 91.67% protection (Fig. 6B). These findings are consistent with those obtained from *in vitro* studies and suggest that vitamin D derivatives potentiated the antiviral activity of T-705 in SFTSV-infected mouse model. In conclusion, the combined drug treatments exhibited increased protective effects compared to the most effective monotherapy.

3.7. Vitamin D derivatives exhibit relatively broad-spectrum antiviral activities

We also explored the antiviral effects of vitamin D derivatives against influenza virus, DENV, and Ross River virus. The EC_{50} values of doxercalciferol against influenza virus strain ZX1109 and Ross River virus were 10.31 ± 5.21 μmol/L and 4.09 ± 0.02 μmol/L, respectively. The EC_{50} value of alfacalcidol against DENV was 2.47 ± 1.57 μmol/L (Table 1). The antiviral activities of doxercalciferol and alfacalcidol were observed in cells under non-cytotoxic conditions. Altogether, these findings demonstratethe broad-spectrum antiviral activity of vitamin D derivatives.

Table 1
The antiviral spectrum of doxercalciferol and alfacalcidol.

Virus	Doxercalciferol		Alfacalcitol	
	EC_{50}[a] (μmol/L)	CC_{50}[b] (μmol/L)	EC_{50}[a] (μmol/L)	CC_{50}[b] (μmol/L)
Influenza virus (ZX1109)	10.31 ± 5.21	25.06 ± 11.36	-[c]	40.57 ± 6.03
DENV	-[c]	11.77 ± 1.91	2.47 ± 1.57	10.79 ± 0.52
Ross River virus	4.09 ± 0.02	11.62 ± 0.13	-[c]	17.69 ± 1.65

[a] Half-maximal effective concentration.
[b] Half-maximal cytotoxic concentration.
[c] Means that EC_{50} cannot be calculated.

4. Discussion

Since SFTSV was first discovered, the virus has posed a significant threat to global public health, as its incidence continues to increase and the distribution of SFTSV transmission continues to expand (Kimet al., 2023), suggesting an urgent need for novel prophylactic and treatment agents.

Vitamin D derivatives have significant potential for treating cancer, hyperparathyroidism, autoimmune diseases, and osteoporosis (Brown et al., 2008; Sintov et al., 2014). To our knowledge, no previous study has examined the anti-SFTSV activity of vitamin D derivatives. Our study has shown that vitamin D derivatives have potent *in vitro* and *in vivo* anti-SFTSV activities. Based on the available results, vitamin D derivatives showed inhibitory activity against all tested viruses, suggesting that it has broad spectrum antiviral activity. To some extent, this may also support the idea that vitamin D derivatives are antiviral through innate immune responses.

Huh7.5 cell line is a variant of the Huh7 cells with RIG-I gene deficiency, while the main difference between BALB/c and A129 mice is that the deficiency of type I interferon receptor in A129 mice. The results of our study indicated that the vitamin D derivatives did not exhibit CPE protective activity in Huh7.5 cells and did not enhance the survival rate or reduce the blood viral load in A129 mice. The induction of IFN-I signaling represents a pivotal event in the antiviral innate immune response. Activation of the RIG-I pathway leads to the expression of IFN. Deficiency in RIG-I results in the suppression of the IFN-I signaling pathway, thereby impeding the initiation of a complete innate immune response. Vitamin D limits Zika virus infection in macrophages and down-regulates chemokines such as CXCL6 (Fernandezet al., 2023). Activation of the stimulator of interferon genes is associated with increased viral gene expression, which typically induces antiviral interferon responses. It is also associated with antiviral TNFSF10 genes (Costa et al., 2024). These findings may provide insight into the antiviral effects of vitamin D derivatives through innate immune regulatory mechanisms.

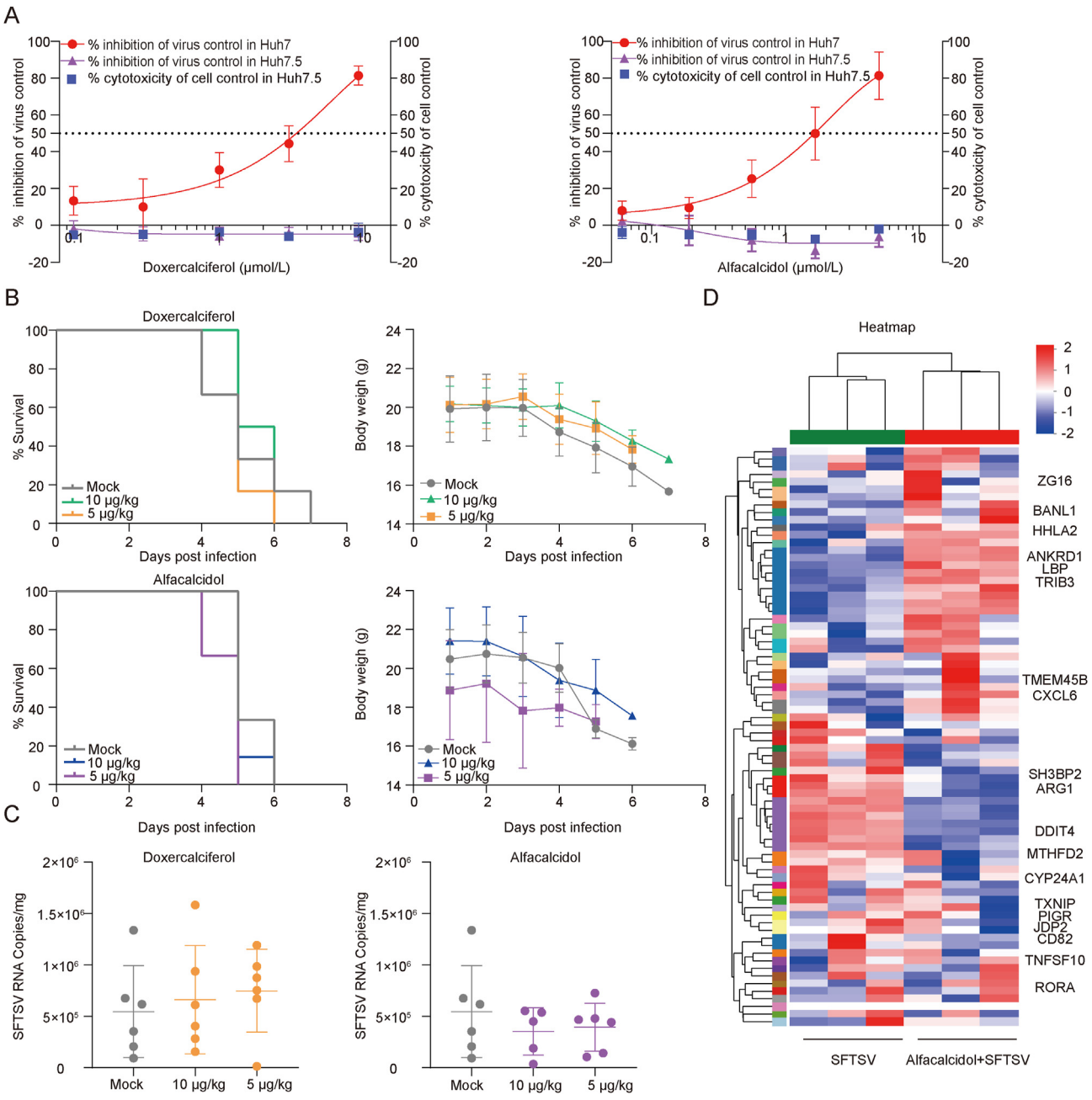

Fig. 5. Mechanism of antiviral action of vitamin D derivatives depends on innate immunity. **A** The antiviral activities of vitamin D derivatives against SFTSV were tested in Huh7 and Huh7.5 cell lines. The inhibition rate and cytotoxicity of antiviral compounds against CPE resulting from SFTSV infection are depicted in red and blue, respectively. **B** The *in vivo* efficacy of vitamin D derivatives in the prophylactic therapy of viremia and survival in 6-8-week-old A129 mice (*Ifnar*$^{-/-}$). Mice were injected intraperitoneal (i.p.) with 10 Plaque forming unit (PFU)/mouse of SFTSV. Meanwhile, mice were administered i.p. with varying doses of vitamin D derivatives. The survival rates and weights of the A129 mice (*Ifnar*$^{-/-}$) were monitored daily for 8 days. **C** The blood of A129 mice (*Ifnar*$^{-/-}$) was assessed at 24 h.p.i., qRT-PCR analysis was used to quantify the viral load in the blood samples. **D** Transcriptome profiles of SFTSV-infected Huh7 cells in the presence and absence of alfacalcidol. A heat map was generated to illustrate the expression profiles of innate immunity differentially expressed genes (DEGs) in all samples. Genes expressed from low to high levels are indicated by colours ranging from blue to red. The left side represents the gene cluster treemap, and the right side represents the sample gene name. The top represents the sample clustering treemap, and the bottom represents the sample name. The six color bars represent three replicates of the experimental and control groups, respectively.

Vitamin D mediates its biological effects by binding to the vitamin D receptor and activating genes and pathways that enhance innate and adaptive immunity (Jiménez-Sousa et al., 2018). Therefore, it is important to use cell and animal models to explore the mechanisms by which vitamin D signaling controls innate immunity. Although this study provides a preliminary investigation of the anti-SFTSV mechanism of vitamin D derivatives and analyzes the potential anti-SFTSV targets associated with vitamin D derivatives in relation to natural immunity based on transcriptomics, further research is warranted to elucidate the specific relevant anti-SFTSV mechanism of action.

Treating SFTSV infection remains a significant challenge in clinical trials. Nevertheless, a novel therapeutic approach is emerging, whereby patients are administered combination drugs. Previous studies have already demonstrated that the combination of T-705 with

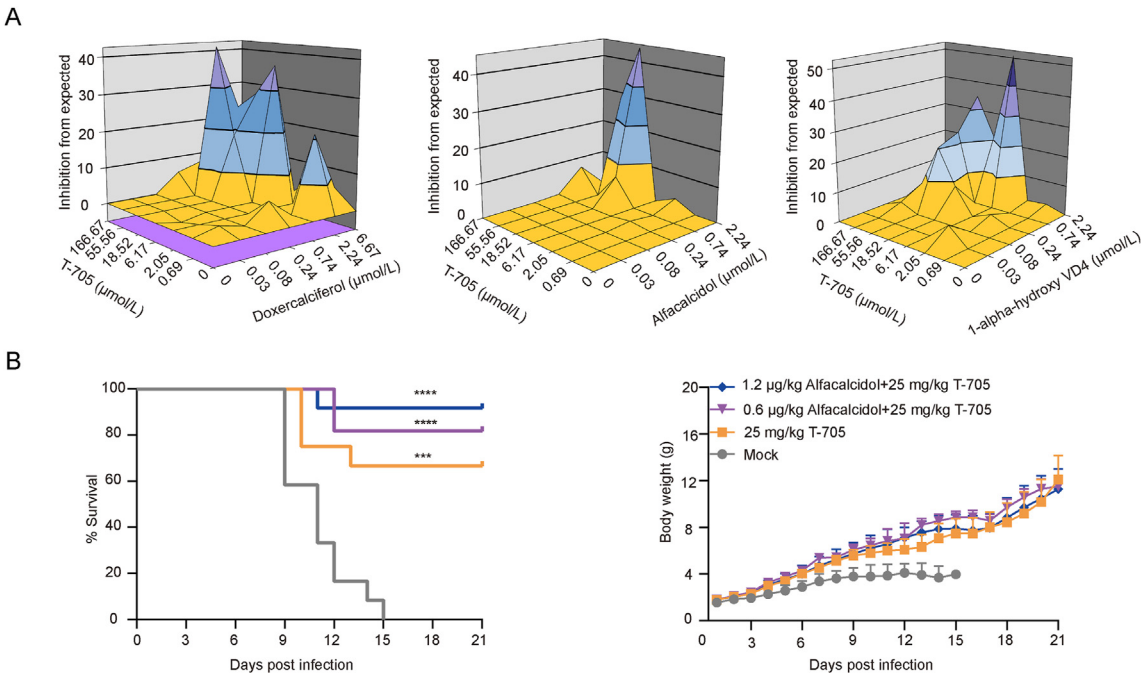

Fig. 6. Combination of vitamin D derivatives and T-705 synergistically inhibited SFTSV infection *in vitro* and *in vivo*. **A** Vitamin D derivatives in combination with the T-705 drug have a high synergistic protective effect at the cellular level. The volumes above and below the planes indicated synergy and antagonism, respectively, with a 95% confidence interval. Synergy analysis was performed using MacSynergy II. **B** The *in vivo* antiviral activity of vitamin D derivatives was evaluated in combination with T-705 using an infectious lethal model in one-day-old ICR suckling mice. Survival curve analysis was processed using the log-rank test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

ribavirin achieves higher efficacy in the treatment of Lassa fever virus and Crimean-Congo haemorrhagic fever virus (Raabe et al., 2017; Singh et al., 2014). Treatment of Rift Valley fever-infected hamsters with a combination of T-705 and ribavirin significantly improved survival rates and reduced viral load in serum and tissue (Scharton et al., 2014). Additionally, the combination of Merimepodib and T-705 has shown synergistic inhibition of Ebola virus replication (Tong et al., 2018). Our study suggests that vitamin D derivatives may enhance the antiviral activities of T-705 to prevent SFTSV infection *in vitro* and *in vivo*. The combination of vitamin D derivatives and T-705 offers a new potential option for clinical treatment in SFTSV patients, and provides a foundation for the future development of a novel therapeutic strategy for the treatment of patients infected with SFTSV.

5. Conclusions

Overall, this study suggests that vitamin D derivatives exhibit potent anti-SFTSV activity *in vitro* and *in vivo*. These results support the use of vitamin D derivatives as candidates for SFTSV prevention. Further studies are required to evaluate their treatment efficacy.

Data availability

All data relevant to the study are included in the article or uploaded as supplementary information.

Ethics statement

All cellular experiments were performed in an enhanced Biosafety Level-2 (BSL-2+) laboratory. All animal experiments were performed in an Animal Biosafety Level-3 (ABSL-3) laboratory. All animal experiments were carried out on the strict basis of ethical guidelines, Animal Welfare Act, and the references of Accreditation of Laboratory Animal Care and other national regulations and rules relating to animals. The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Animal Care and Use Committee of the Beijing Institute of Pharmacology and Toxicology (IACUC-DWZX-2022-622) (Approved on March 14, 2022).

Author contributions

Chong-Da Luo: investigation, data curation, writing-original draft. Xin-Tong Yan: investigation, data curation, revised the manuscript. Shao-Kang Yang: investigation, methodology. *Si*-Chen Ren: investigation, methodology. Yan Luo: investigation, methodology. Jia-Zheng Li: investigation, methodology. Ping Wang: investigation, methodology. Yun-Feng Shao: investigation, methodology. Wei Li: investigation, methodology. Song Li: investigation, methodology. Jing-Jing Yang: Project administration, funding acquisition, writing-review & editing. Rui-Yuan Cao: Project administration, funding acquisition, writing-review & editing. Wu Zhong: Project administration, funding acquisition. All authors have read and agreed to the published version of the manuscript.

Conflict of 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.

Acknowledgements

This research was supported by the National Natural Science Foundation of China (NSFC, grants 81773631, to R.C.; grants 82304383 J.Y.); the National Science and Technology Major Projects for “Major New

Drugs Innovation and Development", China (2018ZX09711003, to W.Z.); the Hainan Provincial Natural Science Foundation of China (822QN299, to J.Y.).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2024.08.007.

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