



RESEARCH ARTICLE

Artesunate and Dihydroartemisinin Inhibit Rabies Virus Replication

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Abstract

Rabies is caused by infection of rabies virus (RABV) and remains a serious threat to the global public health. Except for the requirement for cold chain and high cost of human rabies immune globulin, no small molecule drugs are currently available for clinical treatment of rabies. So, it is of great importance to identify novel compounds that can effectively inhibit RABV infection. Artesunate (ART) and dihydroartemisinin (DHA), two derivatives of artemisinin, are widely used for treatment of malaria in adults and children, showing high safety. In this study, we found that both ART and DHA were able to inhibit RABV replication in host cells at a low concentration (0.1 $\mu\text{mol/L}$). The antiviral effects of ART and DHA were independent of viral strains and cell lines. Pre-treatment with ART or DHA for 2 h *in vitro* did not affect the viral replication in host cells, implying that ART and DHA neither reduced the viability of RABV directly nor inhibited the binding and entrance of the virus to host cells. Further studies revealed that ART and DHA inhibited RABV genomic RNA synthesis and viral gene transcription. Treatment with ART or DHA (5 mg/kg) by intramuscular injection improved, to some extent, the survival rate of RABV-challenged mice. Combination treatment with derivatives of artemisinin and mannitol significantly improved the survival rate of RABV-challenged mice. The results suggest that ART and DHA have a great potential to be explored as new anti-rabies agents for treatment of rabies.

Keywords Rabies virus (RABV) · Artemisinin · Dihydroartemisinin (DHA) · Artesunate (ART) · Antiviral

Introduction

Rabies is an acute zoonotic infectious disease caused by the rabies virus (RABV) that severely impacts the central nervous system. At present, there is no effective drug for treating rabies once clinical symptoms emerge. Rabies can be averted only by effective pre-exposure prophylaxis (PrEP) or post-exposure prophylaxis (PEP) (Franka *et al.*

2009). Most of rabies cases of human come from the bite or scratch by dogs (Fu 1997). Therefore, PrEP is mainly used in individuals who are in close contact with animals. Though rabies in animals can be prevented effectively by rabies vaccination, there are still more than 55,000 rabies-related human deaths annually worldwide (Hampson *et al.* 2015) because of no effective PEP treatment. PEP of rabies involves wound cleaning, rabies vaccination and rabies immune globulin injection. However, rabies immune globulin used for PEP needs cold-chain for transportation and is expensive, so many people in developing countries cannot afford it. In addition, PEP occasionally failed to prevent rabies-related deaths because of nonstandard and delayed treatment. Therefore, there is a great need for development of new cost-effective medications to treat rabies.

A number of antiviral agents against RABV have been reported (Du Pont *et al.* 2019). For instance, favipiravir can significantly inhibit RABV replication *in vitro* and improve the survival rate of RABV-infected mice after treatment (Yamada *et al.* 2016). TMR-001, a proprietary bulk drug

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substance solution of ranpirnase, can inhibit RABV release and cell-to-cell infection *in vitro* (Smith *et al.* 2020). Pymethamine inhibits RABV replication *in vitro* through the inhibition of adenosine synthesis (Rogee *et al.* 2019). *Datura metel* has a strong anti-RABV activity *in vitro* (Roy *et al.* 2016). λ -carrageenan P32 inhibits RABV replication through the inhibition of viral internalization and glycoprotein-mediated cell fusion (Luo *et al.* 2015). Nevertheless, none of them has been approved for clinical treatment of rabies.

Artemisinin and its derivatives (termed artemisinins), such as dihydroartemisinin (DHA) and artesunate (ART), are widely used for treatment of malaria in adults and children, showing high safety (Efferth 2017). Studies have demonstrated that artemisinins not only possess anticancer activity (Odaka *et al.* 2014; Wang *et al.* 2017), but also have antiviral effect (Capci *et al.* 2020; D'Alessandro *et al.* 2020). It has been described that ART inhibits the replication of human cytomegaloviruses in several cell lines (Efferth *et al.* 2002; Schnepf *et al.* 2011). In addition, ART displays a potent antiviral effect against hepatitis B virus (Romero *et al.* 2005) and human herpesvirus-6 (Milbradt *et al.* 2009). Furthermore, ART can also inhibit the replication of Epstein-Barr virus and human JC polyomavirus dose-dependently in cells (Auerochs *et al.* 2011). It has been shown that artemisinin and analogs exert their anti-hepatitis C virus activity by inducing reactive oxygen species (Obeid *et al.* 2013), while artemisinin inhibits the replication of flaviviruses by promoting the type I interferon production (Wang *et al.* 2020). Therefore, artemisinins act as promising antiviral agents through multiple mechanisms.

This study was set to determine whether ART and DHA can act as anti-RABV agents. Here we show that ART and DHA inhibited RABV infection in cells independently of RABV strains and cell lines. Mechanistically, ART and DHA inhibited RABV infection by impeding genomic RNA synthesis and transcription. Treatment with ART or DHA improved the survival rate of RABV-challenged mice. The results support that these anti-malarial drugs may be explored as potential anti-RABV agents.

Material and Methods

Cells, Viruses, Compounds and Animals

Mouse neuroblastoma (NA) cells (Wuhan Institute of Biological Products, Wuhan, China) were cultured in RPMI 1640 medium (Gibco, Suzhou, China) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, New York, USA). Baby hamster kidney (BHK-21) cells (Wuhan Institute of Biological Products, Wuhan, China)

were maintained in Dulbecco's-modified Eagle's medium (DMEM) (Gibco, Suzhou, China) containing 10% FBS (Gibco). RABV challenge virus standard 11 (CVS-11) strain (a gift from Dr. Xianzhu Xia, Academy of Military Medical Sciences, Beijing, China) and rHEP-GFP strain (Luo *et al.* 2016b) were propagated in NA cells. Challenge virus standard 24 (CVS-24) strain (a gift from Dr. Zhenfang Fu, Huazhong Agricultural University, Wuhan, China) was propagated in the brains of suckling mice. GD-SH-01 is a wt RABV strain that was isolated from the brain tissue of rabid pig in our laboratory and is phylogenetically close to canine RABV (Luo *et al.* 2013). Both artesunate (ART) and dihydroartemisinin (DHA) were purchased from TCI America (Portland, OR, USA), and were dissolved in dimethyl sulfoxide (DMSO) for use. Female KM mice (6–7 weeks old) were provided by the Center for Laboratory Animal Science of the Southern Medical University (Guangzhou, China). Mice were housed in the Laboratory Animal Center of the South China Agricultural University.

Cell Viability Assay

The cytotoxicity of ART and DHA in NA and BHK-21 cells was evaluated using the methyl thiazolyl tetrazolium (MTT) assay as described previously (van Tonder *et al.* 2015). Briefly, cell monolayers cultured in 96-well plates were treated with 0–50 $\mu\text{mol/L}$ of ART or DHA for 24 h. Thereafter, MTT assay was performed and the absorbance at 562 nm with a 620 nm reference wavelength was recorded using a Ledetect96 Microplate Reader (Labexim Products, Lengau, Austria). The percentage of cell viability was calculated by comparing the absorbance values of compound-treated groups with those of DMSO-treated groups. The 50% cytotoxic concentration (CC_{50}) was calculated by nonlinear regression (curve fit) using Prism 6 (GraphPad Software, CA, USA). Each experiment was performed in triplicate.

Virus Titration

Virus titers were determined by dFA as described previously (Luo *et al.* 2016a). Briefly, NA cells grown in 96-well cell-culture plates were inoculated with tenfold serial dilutions of the indicated virus in RPMI 1640 medium and incubated for 2 days at 37 °C with 5% CO_2 . Then, the used culture medium was discarded and cells were fixed with 80% acetone for 30 min at – 20 °C. Cells were washed with phosphate-buffered saline (PBS) three times and then stained with FITC-labeled anti-RABV nucleoprotein (N) antibodies (Fujirabio Inc., Malvern, PA, USA) at 37 °C for 60 min. Subsequently, antigen-positive foci were counted under a fluorescence microscope (AMG, Washington, USA) and virus titers were calculated as focus

forming units per milliliter (FFU/mL) using Karber method (Ramakrishnan 2016).

Infection and Compound Treatment Experiments In NA and BHK-21 Cells

NA or BHK-21 cell monolayers cultured in 6-well plates were infected with RABV rHEP-GFP or CVS-11 at a multiplicity of infection (MOI) of 3. After incubation for 1 h at 37 °C with 5% CO₂, supernatants were removed and cells were washed with PBS three times. Next, fresh medium containing 10% FBS and 0.1 μmol/L of ART, DHA or equal volume of DMSO were added. Cells were then incubated at 37 °C with 5% CO₂ for 24 h and supernatants were harvested. Then the cells were stained with FITC-labeled anti-RABV N antibodies and DAPI and fluorescent foci were analyzed under a fluorescence microscope (AMG, Washington, USA). Virus titers of samples were determined in NA cells by dFA, as described above. All titrations were carried out in triplicate.

Assay for the Effects of ART and DHA on RABV's Viability and Entrance

RABV rHEP-GFP and 0.1 μmol/L of ART, DHA or equal volume of DMSO were mixed completely and incubated at 37 °C for 2 h. Then, NA cells cultured in 6-well plates were infected with the above pre-treated RABV at an MOI of 3 as described above. The supernatants were harvested after incubation for 24 h. Virus titers in the cell culture supernatants were determined in NA cells by dFA. Green foci in cells infected with rHEP-GFP were analyzed under a fluorescence microscope.

Quantitative Real-Time PCR

NA cells cultured in 6-well plates were infected with RABV rHEP-GFP at an MOI of 3. After incubation for 1 h at 37 °C, supernatants were removed and cells were washed with PBS three times. Then, fresh medium containing 10% FBS and 0.1 μmol/L of ART, DHA or equal volume of DMSO were added followed by incubation for 24 h at 37 °C. Next, cells were harvested and total RNA was extracted using TRIzol reagent (Invitrogen, Guangzhou, China) according to the manufacturer's protocol. Reverse transcription was performed using the Transcriptor First Strand cDNA Synthesis Kit (Vazyme Biotech Co., Ltd., Nanjing, China). Each reaction was performed in triplicate using SYBR Green Master Mix (Vazyme Biotech Co., Ltd.). Quantitative real-time PCR (qPCR) was performed in a CFX Connect Real-Time Detection System (Bio-Rad, Hercules, CA, USA). The levels of RABV genomic RNA (gRNA) and RABV nucleoprotein (N) mRNA were normalized to

glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers used for qPCR were described previously (Luo *et al.* 2017).

Animal Experiment

KM mice (6–7 weeks of age) were inoculated through intramuscular (i.m.) route with 1.0×10^5 FFU of CVS-11 or GD-SH-01 or 50 MILD₅₀ (mouse intracerebral lethal doses 50) of CVS-24. At 2 and 3 days post inoculation, mice were treated with ART, DHA or PBS by i.m. injection at a dose of 5 mg/kg. The treatment dose referred previous studies (Tan *et al.* 2009; Held *et al.* 2015; Kume *et al.* 2016; Liu *et al.* 2017). Each group contained more than 10 mice. Survival rates were recorded daily for 3 weeks.

Mannitol treatment was performed as described previously (Dufkova *et al.* 2019; Martina *et al.* 2019). Briefly, KM mice (6–7 weeks of age) were inoculated through intramuscular (i.m.) route with 1.0×10^5 FFU of CVS-11. At 2 and 3 days post inoculation, two groups of mice were treated with ART or DHA by i.m. injection at a dose of 5 mg/kg. Two groups of mice were treated with PBS. Thirty minutes after the treatment with compounds, mice were treated intraperitoneally with 500 μL of 25% mannitol in PBS in respective groups except one group treated with PBS. Each group contained 10 mice. Survival rates were recorded daily for 3 weeks.

Statistical Analysis

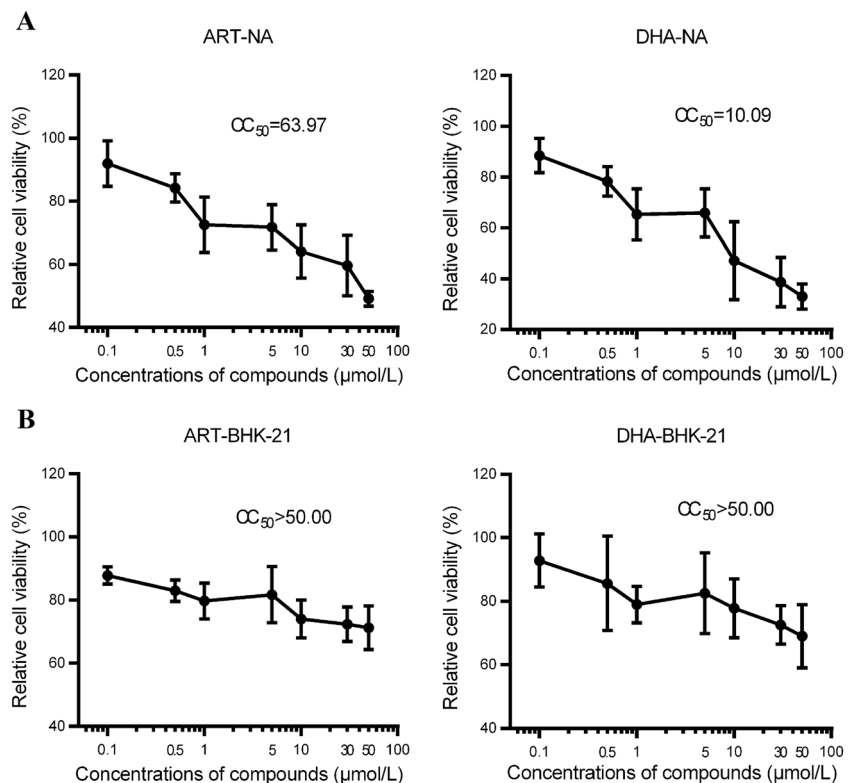
Data were presented as mean values ± standard deviation (SD), and analyzed using Prism 6 (GraphPad Software, San Diego, CA, USA). The statistical significance was determined using Student's *t* test or Logrank Mantel-Cox test. $P < 0.05$ was considered to be significantly different.

Results

ART and DHA Reduces the Cell Viability In A Concentration Dependent Manner

Previous studies have shown that ART and DHA dose-dependently inhibit the proliferation and induce apoptosis of human tumor cells (Odaka *et al.* 2014). Here, we tested whether ART and DHA are cytotoxic in mouse neuroblastoma NA and baby hamster kidney BHK-21 cells. As shown in Fig. 1, 24 h-treatment with ART or DHA decreased the viability of NA and BHK-21 cells in a concentration-dependent manner. The calculated 50% cytotoxicity (CC₅₀) values for ART and DHA were approximately 64 μmol/L and 10 μmol/L in NA cells, respectively, and both more than 50 μmol/L in BHK-21

Fig. 1 ART and DHA reduce the cell viability of NA (A) and BHK-21 (B) cells in a concentration-dependent manner. NA and BHK-21 cells cultured in 96-well plates were treated with DMSO, or 0–50 $\mu\text{mol/L}$ of ART or DHA for 24 h, followed by MTT assay. Absorbance at 562 nm with a 620 nm reference wavelength was recorded. The percentage of cell viability for each treatment was calculated by comparing the absorbance values of compound-treated cells with that of DMSO-treated cells. Each value is expressed as the mean \pm SD. $n = 8$. The 50% cytotoxic concentration (CC_{50}) was calculated by nonlinear regression (curve fit) using GraphPad Prism 6 software.



cells. At 0.1 $\mu\text{mol/L}$, both ART and DHA showed limited cytotoxicity in NA and BHK-21 cells. Hence, this concentration was selected for our *in vitro* RABV replication experiments.

ART and DHA Inhibit the Replication of RABV *In Vitro*

To assess the *in vitro* anti-RABV activity of ART and DHA, NA and BHK-21 cells were infected with RABV rHEP-GFP or CVS-11 (MOI = 3) for 1 h, followed by treatment with DMSO (vehicle control), or 0.1 $\mu\text{mol/L}$ of ART or DHA for 24 h. As shown in Fig. 2A, treatment with either ART or DHA was able to significantly inhibit the release of rHEP-GFP and CVS-11 strains in the culture media of NA cells and BHK-21 cells. As shown in Fig. 2B, treatment with either ART or DHA reduced the expression of RABV N protein in NA cells, indicating inhibition of RABV replication in the cells. Taken together, the results indicate that both ART and DHA were able to inhibit the replication of RABV in host cells.

ART and DHA Neither Reduce the Viability of RABV Directly Nor Inhibit the Entrance of the Virus to Host Cells

Successful replication of RABV in host cells depends on not only the viability of the virus itself, but also the binding

and entering of the virus to host cells (Luo *et al.* 2015). To understand how ART and DHA inhibit the replication of RABV, first of all, we wondered whether ART and DHA reduce the viability of RABV directly and/or inhibit the binding and entrance of the virus to host cells. For this, RABV rHEP-GFP was mixed with ART or DHA (at a final concentration of 0.1 $\mu\text{mol/L}$), or DMSO (vehicle control), followed by incubation at 37 °C for 2 h. Next, NA cells were treated with the above pre-treated RABV rHEP-GFP (MOI = 3) for 1 h at 37 °C. Subsequently, the inoculum was removed, and the cells were washed with PBS three times and re-fed with fresh medium. After 24 h-incubation, the cell culture supernatants were collected, followed by titration of the virus and analysis of green foci formation. We found that pre-incubation of rHEP-GFP with 0.1 $\mu\text{mol/L}$ of ART or DHA for 2 h did not significantly alter the titer of the virus (Fig. 3A), or the expression of GFP in NA cells, compared to pre-incubation with DMSO (Fig. 3B). These results imply that ART and DHA inhibit the replication of RABV neither through directly reducing the viability of RABV nor by interfering with the entrance of the virus to cells.

ART and DHA Inhibit the Synthesis of RABV Genomic RNA and mRNA In Host Cells

RABV is an enveloped, negative-sense, and single-stranded RNA virus (Luo *et al.* 2016b). To understand how

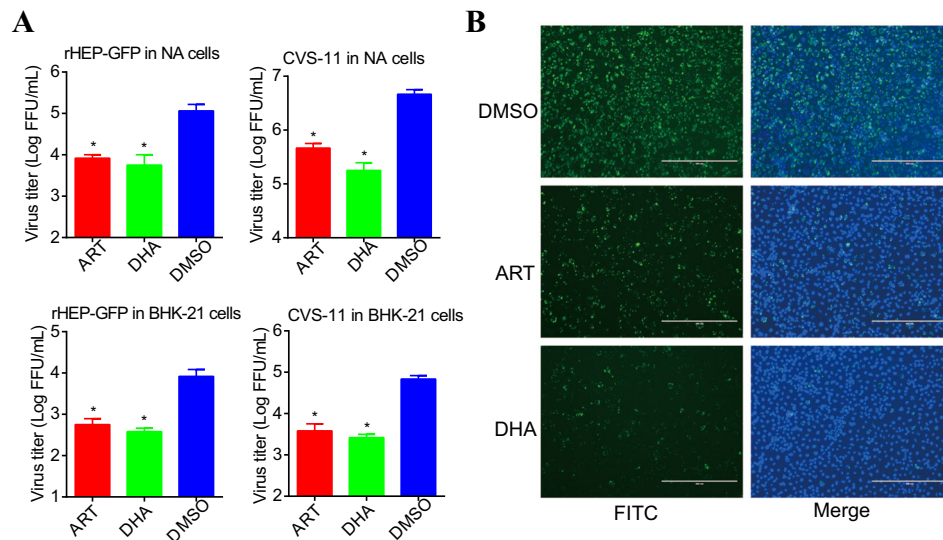


Fig. 2 ART and DHA inhibit the proliferation of RABV in cells. **A** ART and DHA inhibit the release of RABV from cells. NA or BHK-21 cells cultured in 6-well plates were infected with RABV rHEP-GFP or CVS-11 (MOI = 3) for 1 h at 37 °C. Then, the infected cells were treated with DMSO (control), or 0.1 $\mu\text{mol/L}$ of ART or DHA for 24 h at 37 °C. The cell culture supernatants were harvested to determine viral titers in NA cells by dFA, as described above. Titration value is expressed as the mean \pm SD. $n = 3$. Asterisks

indicate significant differences, as calculated by Student's t test ($*P < 0.05$). **B** ART and DHA inhibit the expression of RABV nucleoprotein (N). NA cells were infected with RABV rHEP-GFP (MOI = 3) for 1 h at 37 °C. Then, the infected cells were treated with DMSO (control), or 0.1 $\mu\text{mol/L}$ of ART or DHA for 24 h at 37 °C. Cells were stained with FITC-labeled anti-RABV N antibodies (green) and DAPI (blue) and fluorescent foci were analyzed under a fluorescence microscope (Scale bar: 400 μm).

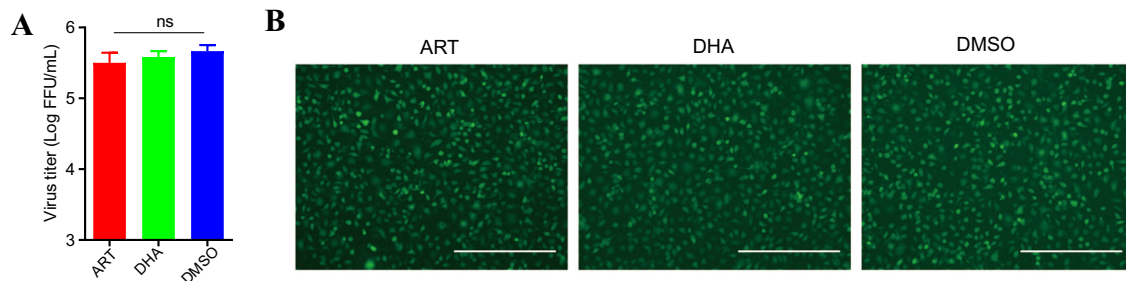


Fig. 3 ART and DHA neither reduce the titer of RABV directly nor interfere with the binding and entrance of RABV to host cells. RABV rHEP-GFP was completely mixed with DMSO (control), or 0.1 $\mu\text{mol/L}$ (final concentration) of ART or DHA, followed by incubation at 37 °C for 2 h. Next, NA cells were treated with the above pre-treated RABV rHEP-GFP (MOI = 3) for 1 h at 37 °C. Then, the inoculum was removed, and the cells were washed with PBS three times and re-

fed with fresh medium containing 10% FBS. Following incubation for 24 h, the cell culture supernatants were harvested. **A** Virus titers of the supernatants were determined in NA cells by dFA. Titration value is expressed as the mean \pm SD. $n = 3$. "ns" indicates non-significant difference, as calculated by Student's t test ($P > 0.05$). **B** Green foci in treated cells were analyzed under a fluorescence microscope (Scale bar: 400 μm).

ART and DHA inhibit RABV replication, firstly we investigated whether the compounds suppress the synthesis of RABV genomic RNA (gRNA) in cells. To this end, NA cells were infected with RABV rHEP-GFP (MOI = 3) for 1 h at 37 °C, followed by 24 h-treatment with ART, DHA or DMSO (vehicle control). Our qPCR analysis showed that treatment with ART or DHA significantly decreased the gRNA level of rHEP-GFP, compared to treatment with DMSO (Fig. 4A). The results indicate that ART and DHA inhibit the the synthesis of RABV genomic RNA.

Next, we studied whether ART and DHA inhibit the transcription of RABV in cells. Nucleoprotein (N) is one of

the gene products of RABV (Luo *et al.* 2016b). By qPCR, we observed that treatment with ART or DHA significantly inhibited the expression of N mRNA in NA cells (Fig. 4B). Decreased transcription might inhibit virus production. Hence, we further determined the ratio of virus/genomic-RNA. Of interest, treatment with ART or DHA did decrease the ratio of virus/genomic-RNA (Fig. 4C). The results demonstrated that ART and DHA inhibit RABV replication through repressing its genomic RNA synthesis and transcription.

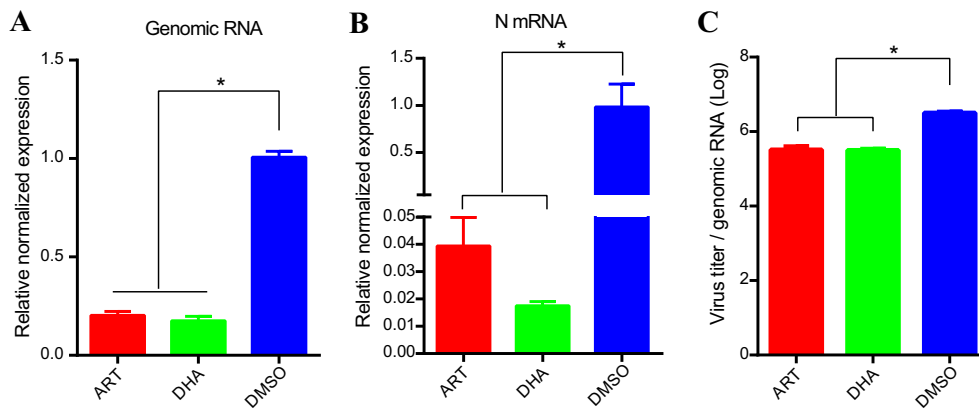


Fig. 4 ART and DHA inhibit the synthesis of RABV genomic RNA and mRNA in cells. NA cells were infected with RABV rHEP-GFP (MOI = 3) for 1 h at 37 °C. Then, the infected cells were treated with DMSO (control), or 0.1 $\mu\text{mol/L}$ of ART or DHA for 24 h at 37 °C. qPCR was performed to determine the expression of RABV genomic

RNA (gRNA) and RABV N mRNA. The levels of gRNA (A) and N mRNA (B) were normalized to GAPDH. C Virus/genomic-RNA ratio was calculated based on the levels of gRNA and virus titer. The results represent the mean \pm SD. $n = 3$. Asterisks indicate significant differences as calculated by Student's t test ($*P < 0.05$).

Treatment with ART or DHA Improves the Survival Rate of Lethal RABV-Challenged Mice

To test whether ART or DHA has a therapeutic potential for rabies, mice were challenged with pathogenic strains CVS-11, GD-SH-01 and CVS-24, and then treated with ART or DHA (5 mg/kg), or PBS (control) by i.m. injection at 2 and 3 days post-infection. We chose the time points of compound treatment considering two points: one is to wait for RABV to invade into the CNS, and the second is to check the efficacy of treatment after 48 h of infection with RABV. As shown in Fig. 5, ART treatment modestly (by 25%) but not significantly increased the survival rate of CVS-11, CVS-24 or GD-SH-01 challenged mice, compared to PBS treatment. DHA treatment also modestly increased the survival rate of mice infected with CVS-11, but not that of the animals infected with CVS-24 and GD-SH-01. The results indicate that i.m. route treatment with ART or DHA has certain antiviral effect against RABV *in vivo*.

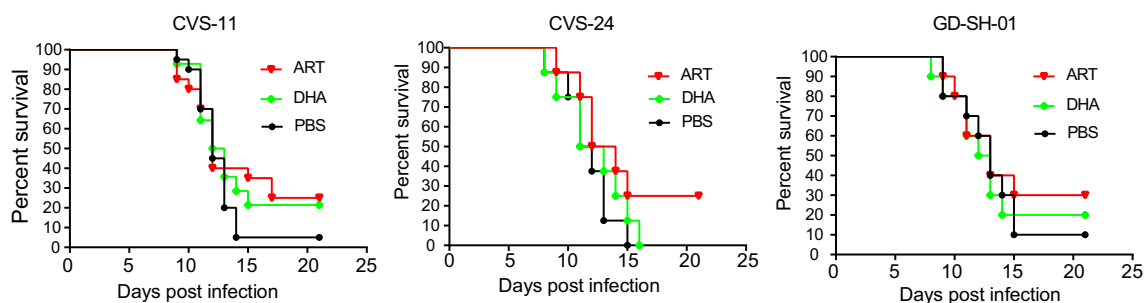


Fig. 5 ART and DHA improve the survival rate of lethal RABV-challenged mice. KM mice were infected with 1.0×10^5 FFU of CVS-11, GD-SH-01 or 50 MILD₅₀ of CVS-24 through intramuscular (i.m.) route. At 2 and 3 days post infection, mice were treated with

Combination Treatment with Derivatives of Artemisinin and Mannitol Significantly Improves the Survival Rate of Lethal RABV-Challenged Mice

Intraperitoneal administration of 500 μL of 25% mannitol was found to represent an effective dose for the enhancement of blood-brain barrier (BBB) permeability and has no visible effects in mice (Louboutin *et al.* 2010; Dufkova *et al.* 2019). To make compounds preferably passed through BBB, CVS-11 infected mice were treated with combination drugs (ART+ mannitol; DHA+ mannitol). As shown in Fig. 6, mannitol treatment alone modestly but not significantly increased the survival rate of infected mice. Combination treatment with ART or DHA and mannitol significantly improved the survival rate of challenged mice (Fig. 6).

PBS (control), or ART or DHA by i.m. injection at a dose of 5 mg/kg. Survival rates were recorded daily for 3 weeks. The results represent the mean \pm SD. $n = 10$. Data are analyzed by Logrank Mantel-Cox test ($P > 0.05$).

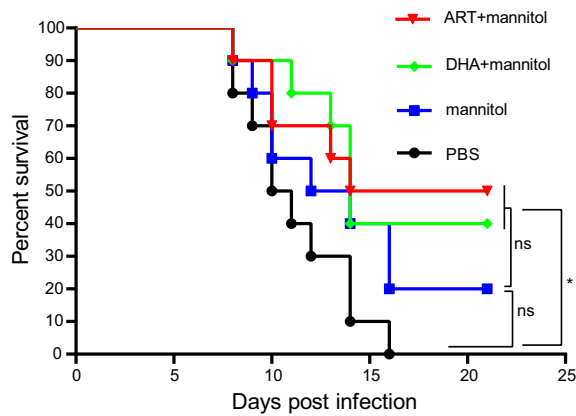


Fig. 6 Mannitol treatment enhances the inhibitory effect of ART and DHA on CVS-11. KM mice were infected with 1.0×10^5 FFU of CVS-11 through i.m. route. At 2 and 3 days post infection, mice were treated with ART, DHA or PBS. Thirty minutes after the treatment with compounds, mice were treated intraperitoneally with 25% mannitol in respective groups. Survival rates were recorded daily for 3 weeks. The results represent the mean \pm SD. $n = 10$. Data are analyzed by Logrank Mantel-Cox test (* $P < 0.05$, ns: non-significant differences).

Discussion

In the present study, for the first time, we present evidence that anti-malarial drugs ART and DHA possessed antiviral activity against RABV *in vitro* (in cells) and *in vivo* (in mice). In this study, normal baby hamster kidney fibroblasts (BHK-21) were found to be highly resistant to DHA or ART ($CC_{50} > 50 \mu\text{mol/L}$), which is in line with the previous findings (Odaka *et al.* 2014), supporting the high safety profiles of artemisinins (Efferth 2017). Since ART or DHA at $0.1 \mu\text{mol/L}$ (100 nmol/L) had limited cytotoxicity in both NA and BHK-21 cells, we selected this concentration for our *in vitro* studies, in order to minimize the cytotoxic effect on RABV replication in the two cell lines. Both ART and DHA at such a nanomolar concentration were able to inhibit the replication of rHEP-GFP and CVS-11 strains in NA and BHK-21 cells, indicating that ART or DHA can potently inhibit RABV replication independently of viral strains and cell lines.

Previous studies have described multiple mechanisms of action of anti-RABV agents, such as inhibition of RABV internalization (Luo *et al.* 2015), inhibition of adenosine synthesis (Rogee *et al.* 2019), and inhibition of RABV release (Smith *et al.* 2020). In addition, ribavirin, a broad-spectrum guanosine nucleoside analog, inhibits the virus infection by inhibiting mRNA expression (Kihira *et al.* 2014; Musser *et al.* 2015; Aljabr *et al.* 2016), which may be due to inhibition of mRNA capping (Kentsis *et al.* 2004). In this study, we observed that pre-treatment with $0.1 \mu\text{mol/L}$ of ART or DHA for 2 h *in vitro* did not affect the viral replication in host cells, implying that ART and DHA neither reduced the viability of RABV directly nor inhibited the

entrance of the virus to host cells. However, treatment with ART or DHA profoundly inhibited the genomic RNA synthesis and N mRNA transcription of RABV, leading to inhibition of RABV replication. ART-mediated inhibition of viral gene expression has been documented in human cytomegaloviruses, herpesviruses and Epstein–Barr virus (Efferth *et al.* 2002; Milbradt *et al.* 2009; Auerochs *et al.* 2011). Therefore, here we propose that ART and DHA may execute the antiviral action by blocking the viral gene expression of RABV, thereby inhibiting the synthesis of viral proteins and consequently preventing the onset of subsequent steps of the viral lytic replication cycle.

In this study, we noticed that ART and DHA were able to potently inhibit RABV infection in cell culture, but only modestly improved the survival rate of RABV-infected mice. Blood–brain barrier (BBB) blocks the delivery of some antiviral drugs and molecules to the central nervous system, which increases the difficulties to treat clinical rabies (Smith *et al.* 2019). We speculate that the weak *in vivo* anti-RABV effect of ART and DHA might be due to the fact that only limited amount of the compounds was passing through BBB after i.m. injection. Furthermore, ART can ameliorate BBB breakdown in malaria infection in mice (Souza *et al.* 2012; Du *et al.* 2017), which is not beneficial to RABV clearance (Roy *et al.* 2007; Roy and Hooper 2007). As a BBB opener, mannitol helped ART or DHA to improve the survival rate of RABV-infected mice. Therefore, enhanced BBB permeability boosts the inhibitory effect of ART or DHA on RABV. Previous studies have shown that artemisinin can pass through BBB through intranasal administration (Marignon *et al.* 2014; Long *et al.* 2020). It is worthy to test whether intranasal administration of ART or DHA is an effective therapeutic route for rabies. In addition, besides BBB permeability, challenge doses of lethal RABV may also weaken the curative effect of antiviral drug (Banyard *et al.* 2019). Favipiravir shows different anti-RABV effects in separate studies, which may be related to different RABV strains, different challenge doses or different routes of administration used (Yamada *et al.* 2016; Banyard *et al.* 2019). In this study, mice were inoculated (i.m.) with 1.0×10^5 FFU of CVS-11, GD-SH-01 or 50 $MILD_{50}$ of CVS-24. We treated the RABV challenged mice with ART or DHA at a dose of 5 mg/kg by i.m. injection at 2 and 3 days post-infection. Further studies are required to determine the challenge doses/strains of RABV and the administration doses/routes/time points of drugs to improve the therapy effect.

In this study, treatment with ART improved the survival rate of CVS-11, CVS-24 or GD-SH-01 challenged mice, whereas treatment with DHA only improved the survival rate of CVS-11 challenged mice. DHA is an active metabolite of all artemisinins, including ART (Efferth 2017). It is unclear why ART displayed better therapeutic effect than DHA. Recently we have observed that ART could enhance the immune response of inactivated rabies vaccines (Luo *et al.* 2019). In addition, artemisinin-induced antiviral effect

against flaviviruses is associated with enhanced host type I interferon response (Wang *et al.* 2020). Our previous studies have demonstrated that clearance of infected RABV needs boosted immune response (Luo *et al.* 2020a, b). In addition, the molecular weight of ART is 384.43 and the molecular weight of DHA is 284.35. However, ART is hydrosoluble while DHA is liposoluble. Likely, the improvement of the survival rate of GD-SH-01 and CVS-24 challenged mice by ART may be partly attributed to its better absorption and enhanced immune response in mice.

Increasing evidence indicates that a combination therapy with two-to-three drugs can exhibit better anti-RABV effect than a single drug therapy (Marosi *et al.* 2019; Smreczak *et al.* 2019). In particular, combination of ART with ganciclovir, foscarnet or cidofovir can result in moderate synergism in human cytomegalovirus infections (Drouot *et al.* 2016). To reposition ART or DHA for treatment of rabies, it would be interesting to assess whether ART or DHA is synergistic or additive with other antiviral drugs.

In summary, here we have shown that artemisinin derivatives ART and DHA possess anti-RABV activity *in vitro* and *in vivo*. Both of them can inhibit RABV genomic RNA synthesis and viral gene transcription. This study provides the first evidence that these two anti-malarial drugs have a great potential as anti-RABV agents.

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Author Contributions XG, JL and SH conceived and designed the experiments. JL, YZ, YW, QL, JL and HH performed the experiments. JL, XG and SH analyzed the data. JL and YL wrote the manuscript and prepared the figures. XG and SH checked and finalized the manuscript. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare no conflict of interest.

Animal and Human Rights Statement All animal experiments were approved by the Ethics Committee for Animal Experiments of the South China Agricultural University (ethics committee approval number: 2019014), and conducted in compliance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People's Republic of China.

References

- Aljabr W, Touzelet O, Pollakis G, Wu W, Munday DC, Hughes M, Hertz-Fowler C, Kenny J, Fearn R, Barr JN, Matthews DA, Hiscox JA (2016) Investigating the influence of ribavirin on human respiratory syncytial virus RNA synthesis by using a high-resolution transcriptome sequencing approach. *J Virol* 90:4876–4888
- Auerochs S, Korn K, Marschall M (2011) A reporter system for Epstein–Barr virus (EBV) lytic replication: anti-EBV activity of the broad anti-herpesviral drug artesunate. *J Virol Methods* 173:334–339
- Banyard AC, Mansfield KL, Wu G, Selden D, Thorne L, Birch C, Koraka P, Osterhaus A, Fooks AR (2019) Re-evaluating the effect of Favipiravir treatment on rabies virus infection. *Vaccine* 37:4686–4693
- Capci A, Lorion MM, Mai C, Hahn F, Hodek J, Wangen C, Weber J, Marschall M, Ackermann L, Tsogoeva SB (2020) (Iso)Quinoline-Artemisinin hybrids prepared through click chemistry: highly potent agents against viruses. *Chemistry* 26:12019–12026
- D'Alessandro S, Scaccabarozzi D, Signorini L, Perego F, Ilboudo DP, Ferrante P, Delbue S (2020) The use of antimalarial drugs against viral infection. *Microorganisms* 8:85
- Drouot E, Piret J, Boivin G (2016) Artesunate demonstrates in vitro synergism with several antiviral agents against human cytomegalovirus. *Antivir Ther* 21:535–539
- Du Pont V, Plemper RK, Schnell MJ (2019) Status of antiviral therapeutics against rabies virus and related emerging lyssaviruses. *Curr Opin Virol* 35:1–13
- Du Y, Chen G, Zhang X, Yu C, Cao Y, Cui L (2017) Artesunate and erythropoietin synergistically improve the outcome of experimental cerebral malaria. *Int Immunopharmacol* 48:219–230
- Dufkova L, Sirmarova J, Salat J, Honig V, Palus M, Ruzek D, Fooks AR, Mansfield KL, Tordo N, Jochmans D, Neyts J, Martina B, Koraka P, Osterhaus ADME (2019) Mannitol treatment is not effective in therapy of rabies virus infection in mice. *Vaccine* 37:4710–4714
- Efferth T (2017) From ancient herb to modern drug: artemisia annua and artemisinin for cancer therapy. *Semin Cancer Biol* 46:65–83
- Efferth T, Marschall M, Wang X, Huong SM, Hauber I, Olbrich A, Kronschnabl M, Stamminger T, Huang ES (2002) Antiviral activity of artesunate towards wild-type, recombinant, and ganciclovir-resistant human cytomegaloviruses. *J Mol Med (Berl)* 80:233–242
- Franka R, Wu X, Jackson FR, Velasco-Villa A, Palmer DP, Henderson H, Hayat W, Green DB, Blanton JD, Greenberg L, Rupprecht CE (2009) Rabies virus pathogenesis in relationship to intervention with inactivated and attenuated rabies vaccines. *Vaccine* 27:7149–7155
- Fu ZF (1997) Rabies and rabies research: past, present and future. *Vaccine* 15(Suppl):S20–S24
- Hampson K, Coudeville L, Lembo T, Sambo M, Kieffer A, Atatlan M, Barrat J, Blanton JD, Briggs DJ, Cleaveland S, Costa P, Freuling CM, Hiby E, Knopf L, Leanes F, Meslin F, Metlin A, Miranda ME, Mueller T, Nel LH, Recuenco S, Rupprecht CE, Schumacher C, Taylor L, Natal Vigilato MA, Zinsstag J, Dushoff J (2015) Estimating the global burden of endemic canine rabies. *PLoS Negl Trop Dis* 9:e0003709
- Held J, Supan C, Salazar CLO, Tinto H, Bonkian LN, Nahum A, Moulou B, Sie A, Coulibaly B, Sirima SB, Siribie M, Otsyula N, Otieno L, Abdallah AM, Kimutai R, Bouyou-Akotet M, Kombila M, Koiwai K, Cantalloube C, Din-Bell C, Djeriou E, Waitumbi J, Mordmueller B, Ter-Minassian D, Lell B, Kremsner PG (2015) Ferroquine and artesunate in African adults and children with *Plasmodium falciparum* malaria: a phase 2, multicentre, randomised, double-blind, dose-ranging, non-inferiority study. *Lancet Infect Dis* 15:1409–1419
- Kentsis A, Topisirovic I, Culjkovic B, Shao L, Borden KL (2004) Ribavirin suppresses eIF4E-mediated oncogenic transformation by physical mimicry of the 7-methyl guanosine mRNA cap. *Proc Natl Acad Sci USA* 101:18105–18110

- Kihira S, Uematsu J, Kawano M, Itoh A, Ookohchi A, Satoh S, Maeda Y, Sakai K, Yamamoto H, Tsurudome M, O'Brien M, Komada H (2014) Ribavirin inhibits human parainfluenza virus type 2 replication *in vitro*. *Microbiol Immunol* 58:628–635
- Kume A, Dang TMA, Shichiri M, Ishida N, Suzuki H (2016) Probucol dramatically enhances dihydroartemisinin effect in murine malaria. *Malar J* 15:472
- Liu J, Hong X, Lin D, Luo X, Zhu M, Mo H (2017) Artesunate influences Th17/Treg lymphocyte balance by modulating Treg apoptosis and Th17 proliferation in a murine model of rheumatoid arthritis. *Exp Ther Med* 13:2267–2273
- Long Y, Yang Q, Xiang Y, Zhang Y, Wan J, Liu S, Li N, Peng W (2020) Nose to brain drug delivery—a promising strategy for active components from herbal medicine for treating cerebral ischemia reperfusion. *Pharmacol Res* 159:104795
- Louboutin J, Chekmasova AA, Marusich E, Chowdhury JR, Strayer DS (2010) Efficient CNS gene delivery by intravenous injection. *Nat Methods* 7:905–909
- Luo Y, Zhang Y, Liu X, Yang Y, Yang X, Zheng Z, Deng X, Wu X, Guo X (2013) Characterization of a wild rabies virus isolate of porcine origin in China. *Infect Genet Evol* 17:147–152
- Luo Z, Tian D, Zhou M, Xiao W, Zhang Y, Li M, Sui B, Wang W, Guan H, Chen H, Fu ZF, Zhao L (2015) lambda-carrageenan P32 is a potent inhibitor of rabies virus infection. *PLoS ONE* 10:e140586
- Luo J, Shi H, Tan Y, Niu X, Long T, Zhao J, Tian Q, Wang Y, Chen H, Guo X (2016a) Two potential recombinant rabies vaccines expressing canine parvovirus virion protein 2 induce immunogenicity to canine parvovirus and rabies virus. *Vaccine* 34:4392–4398
- Luo J, Zhao J, Tian Q, Mo W, Wang Y, Chen H, Guo X (2016b) A recombinant rabies virus carrying GFP between N and P affects viral transcription *in vitro*. *Virus Genes* 52:379–387
- Luo J, Zhang B, Wu Y, Tian Q, Zhao J, Lyu Z, Zhang Q, Mei M, Luo Y, Guo X (2017) Expression of interleukin-6 by a recombinant rabies virus enhances its immunogenicity as a potential vaccine. *Vaccine* 35:938–944
- Luo J, Zhang Y, He H, Liu Q, Huang S, Guo X (2019) Artesunate enhances the immune response of rabies vaccine as an adjuvant. *Vaccine* 37:7478–7481
- Luo J, Zhang B, Lyu Z, Wu Y, Zhang Y, Guo X (2020a) Single amino acid change at position 255 in rabies virus glycoprotein decreases viral pathogenicity. *Faseb J* 34:9650–9663
- Luo J, Zhang B, Wu Y, Guo X (2020b) Amino acid mutation in position 349 of glycoprotein affect the pathogenicity of rabies virus. *Front Microbiol* 11:481
- Marijon A, Bonnot G, Fourier A, Bringer C, Lavoignat A, Gagnieu MC, Bienvenu AL, Picot S (2014) Efficacy of intranasal administration of artesunate in experimental cerebral malaria. *Malar J* 13:501
- Marosi A, Dufkova L, Forro B, Felde O, Erdelyi K, Sirmarova J, Palus M, Honig V, Salat J, Tikos R, Gyuranecz M, Ruzek D, Martina B, Koraka P, Osterhaus A, Bakonyi T (2019) Combination therapy of rabies-infected mice with inhibitors of pro-inflammatory host response, antiviral compounds and human rabies immunoglobulin. *Vaccine* 37:4724–4735
- Martina BEE, Smreczak M, Orłowska A, Marzec A, Trebas P, Roose JM, Zmudzinski J, Gerhauser I, Wohlsein P, Baumgaertner W, Osterhaus ADME, Koraka P (2019) Combination drug treatment prolongs survival of experimentally infected mice with silver-haired bat rabies virus. *Vaccine* 37:4736–4742
- Milbradt J, Auerochs S, Korn K, Marschall M (2009) Sensitivity of human herpesvirus 6 and other human herpesviruses to the broad-spectrum anti-infective drug artesunate. *J Clin Virol* 46:24–28
- Musser JM, Heatley JJ, Koinis AV, Suchodolski PF, Guo J, Escandon P, Tizard IR (2015) Ribavirin inhibits parrot bornavirus 4 replication in cell culture. *PLoS ONE* 10:e134080
- Obeid S, Alen J, Nguyen VH, Pham VC, Meuleman P, Pannecouque C, Le TN, Neyts J, Dehaen W, Paeshuyse J (2013) Artemisinin analogues as potent inhibitors of *in vitro* hepatitis C virus replication. *PLoS ONE* 8:e81783
- Odaka Y, Xu B, Luo Y, Shen T, Shang C, Wu Y, Zhou H, Huang S (2014) Dihydroartemisinin inhibits the mammalian target of rapamycin-mediated signaling pathways in tumor cells. *Carcinogenesis* 35:192–200
- Ramakrishnan MA (2016) Determination of 50% endpoint titer using a simple formula. *World J Virol* 5:85–86
- Rogee S, Larrous F, Jochmans D, Ben-Khalifa Y, Neyts J, Bourhy H (2019) Pyrimethamine inhibits rabies virus replication *in vitro*. *Antivir Res* 161:1–9
- Romero MR, Efferth T, Serrano MA, Castano B, Macias RI, Briz O, Marin JJ (2005) Effect of artemisinin/artesunate as inhibitors of hepatitis B virus production in an “*in vitro*” replicative system. *Antivir Res* 68:75–83
- Roy A, Hooper DC (2007) Lethal silver-haired bat rabies virus infection can be prevented by opening the blood-brain barrier. *J Virol* 81:7993–7998
- Roy A, Phares TW, Koprowski H, Hooper DC (2007) Failure to open the blood-brain barrier and deliver immune effectors to central nervous system tissues leads to the lethal outcome of silver-haired bat rabies virus infection. 1110–1118
- Roy S, Mukherjee S, Pawar S, Chowdhary A (2016) Evaluation of *In vitro* Antiviral Activity of Datura metel Linn. Against rabies virus. *Pharmacogn Res* 8:265–269
- Schnepf N, Corvo J, Pors MJ, Mazon MC (2011) Antiviral activity of ganciclovir and artesunate towards human cytomegalovirus in astrocytoma cells. *Antivir Res* 89:186–188
- Smith SP, Wu G, Fooks AR, Ma J, Banyard AC (2019) Trying to treat the untreatable: experimental approaches to clear rabies virus infection from the CNS. *J Gen Virol* 100:1171–1186
- Smith TG, Jackson FR, Morgan CN, Carson WC, Martin BE, Gallardo-Romero N, Ellison JA, Greenberg L, Hodge T, Squiquera L, Sulley J, Olson VA, Hutson CL (2020) Antiviral ranpirnase TMR-001 inhibits rabies virus release and cell-to-cell infection *in vitro*. *Viruses* 12:177
- Smreczak M, Orłowska A, Marzec A, Trebas P, Kycko A, Reichert M, Koraka P, Osterhaus A, Zmudzinski JF (2019) The effect of combined drugs therapy on the course of clinical rabies infection in a murine model. *Vaccine* 37:4701–4709
- Souza MC, Paixao FH, Ferraris FK, Ribeiro I, Henriques M (2012) Artesunate exerts a direct effect on endothelial cell activation and NF-kappaB translocation in a mechanism independent of plasmodium killing. *Malar Res Treat* 2012:679090
- Tan B, Naik H, Jang I, Yu K, Kirsch LE, Shin C, Craft JC, Fleckenstein L (2009) Population pharmacokinetics of artesunate and dihydroartemisinin following single- and multiple-dosing of oral artesunate in healthy subjects. *Malar J* 8:304
- van Tonder A, Joubert AM, Cromarty AD (2015) Limitations of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay when compared to three commonly used cell enumeration assays. *BMC Res Notes* 8:47
- Wang L, Liu L, Wang J, Chen Y (2017) Inhibitory effect of artesunate on growth and apoptosis of gastric cancer cells. *Arch Med Res* 48:623–630
- Wang X, Zheng B, Ashraf U, Zhang H, Cao C, Li Q, Chen Z, Imran M, Chen H, Cao S, Ye J (2020) Artemisinin inhibits the replication of flaviviruses by promoting the type I interferon production. *Antivir Res* 179:104810
- Yamada K, Noguchi K, Komeno T, Furuta Y, Nishizono A (2016) Efficacy of favipiravir (T-705) in rabies postexposure prophylaxis. *J Infect Dis* 213:1253–1261