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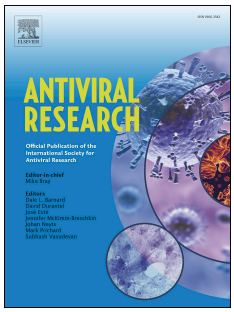

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Micafungin is a novel anti-viral agent of chikungunya virus through multiple mechanisms

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Abstract

The chikungunya virus (CHIKV) is a mosquito-borne virus that belongs to the genus *Alphavirus*, family *Togaviridae*. It is the cause of chikungunya fever in humans, which presents a serious global threat due to its high rate of contagion. The clinical symptoms of CHIKV include fever and persistent, severe arthritis. Micafungin has broad-spectrum fungicidal activity against *Candida* spp. is a promising echinocandin that was recently approved by the U.S. Food and Drug Administration (FDA) and has demonstrated activity against *Candida* and *Aspergillus*. Recent studies have demonstrated the antiviral activity of micafungin; however, the inhibitory effects against CHIKV have yet to be investigated. Our objectives in this study were to explore the antiviral effects of micafungin on CHIKV infection and to elucidate the potential molecular mechanisms of inhibition. We determined that micafungin has the ability to counter CHIKV-induced cytopathic effects. We further discovered that micafungin limits virus replication, release, cell-to-cell transmission, and also slightly affected virus stability during high doses treatment. The efficacy of micafungin was further confirmed against two clinical isolates of CHIKV and

two alphaviruses: Sindbis virus (SINV) and Semliki Forest virus (SFV). Our findings suggest that micafungin has considerable potential as a novel inhibitor against the viral replication, and intracellular and extracellular transmission of CHIKV, and has a little effect on virus stability. Our findings also suggest that micafungin could have curative effects on other alphavirus infections.

Keywords: Antiviral, Chikungunya virus, Micafungin, Replication, Transmission

1. Introduction

The chikungunya virus (CHIKV) is a mosquito-borne virus that belongs to the genus *Alphavirus*, family *Togaviridae*. It has been reported that 72~97% of those become infected with CHIKV develop chikungunya fever, which presents as a fever-rash-arthralgia syndrome (causing fever, joint pain, muscle pain, headache, joint swelling, and a rash) and can lead to debilitating pain that lasts for several months to a year (Weaver and Lecuit, 2015). In the 1950s, CHIKV was isolated from Africa (Weaver and Lecuit, 2015), but the virus has since spread across the Asia, Europe, and Americas, with millions of people becoming infected during recent years (Delisle et al., 2015; Wahid et al., 2017). The incidence of CHIKV is a serious threat to global health, and CHIKV has been listed as a priority pathogen by the National Institutes of Allergy and Infectious Diseases (NIAID) in the US (Rougeron et al., 2015; Weaver and Lecuit, 2015).

As of yet, there are no licensed antivirals and vaccines available for chikungunya disease, due to the fact that current therapeutic strategies are

based on the alleviation of disease symptoms (Powers, 2018; Rougeron et al., 2015; Weaver and Lecuit, 2015). Therefore, the discovery and development of new antiviral drugs is an important goal in the fight to counter CHIKV-induced disease.

Micafungin is a large lipopeptide molecule with a complex aromatic side chain (Iwamoto et al., 1994). This molecule inhibits the synthesis of fungal cell walls by targeting the β-1,3-D-glucan synthase enzyme of fungi (Douglas, 2001). Micafungin is classified as a member of the family of echinocandin. It has been approved drug for *Candida* infection by the U.S. Food and Drug Administration (FDA) (Iwamoto et al., 1994). Indeed, micafungin has been shown to inhibit the activity of *Candida* species and is licensed as a first-line treatment for invasive candidiasis *in-vitro* and *in-vivo* (Martial et al., 2017; Pappas et al., 2016). Micafungin and other echinocandins have achieved adequate clinical responses in approximately 70% of invasive candidiasis or candidaemia cases (Andes et al., 2012; Kullberg and Arendrup, 2015). At subinhibitory concentrations, micafungin induces a re-routing of metabolic pathways, which limits protein synthesis and cell replication in *Candida albicans* (Katragkou et al., 2017). In a phase III clinical trial study of patients, micafungin has been defined the clinical pharmacodynamic (PD) target required for micafungin efficacy for invasive candidiasis or candidemia and suggest differences in the concentration-time curve (AUC)/MIC ratio target among *Candida* species (Andes et al., 2011). The formulation of micafungin is powder for intravenous solution and the daily recommended doses are 12.5-869 mg for adult patients. Micafungin with linear PK over a dose range of 0.15–8 mg/kg/day, C_{max} values of micafungin were about 12~16 mg/L

(equivalent 12~16 μg/ml) under the daily 150 mg dose infused in 60 min. The AUC after a single dose and at steady state were 116 mg h/L and 181.5 mg h/L. Micafungin possesses the highly plasma protein binding (99.8%). In health adult, the clearance (CL) is 10.4 mL/h/kg and the volume of distribution is 0.2 L/kg. In clinical treatment, the favorable dose of micafungin that can safely be administered is a daily (QD) intravenous infusion of 100~200 mg (Martial et al., 2017). One recent publication reported that micafungin is an effective agent against the proliferation of enterovirus 71 (EV71) and possesses antiviral activity against other enteroviruses, such as coxsackievirus group B type 3 (CVB3) and human rhinovirus (HRV) (Kim et al., 2016)

In this study, we (1) determined that micafungin is an effective anti-CHIKV drug in U2OS cells infected with CHIKV S27 and (2) investigated the mechanism by which micafungin disrupts CHIKV infection. Our results also indicated that micafungin may have use as a broad spectrum treatment against other alphavirus infections, such as SINV and SFV. This is the first study to report on the anti-CHIKV properties of micafungin.

2. Materials and Methods

2.1 Viruses, cells, and compounds

CHIKV S27(ATCC-VR-64), two clinical strains of CHIKV (0611aTw and 0810bTw [had a E1-226V mutant]), SINV (ATCC-VR-68), and SFV (ATCC-VR-67) were investigated in this study. The viruses were propagated in BHK cells and titrated using $TCID_{50}$ assays. U2OS cells (ATCC: HTB-96) and BHK-21 cells (ATCC: CCL-10) were cultured in DMEM (Biological Industries, 01-052-1) with 12.5 mM HEPES (Biological Industries, 03-025-1B),

L-Alanyl-L-Glutamine (Biological Industries, 03-022-1B), antibiotics (Biological Industries, 03-033-1B), and 5% fetal bovine serum (Biological Industries, 04-001-1) at 37℃ under 5% CO_2. Micafungin (Selleckchem, 208538-73-2) and suramin (Sigma-Aldrich, S2671) were purchased and dissolved as a stock for experiments.

2.2 RT-qPCR

The total RNA of treated U2OS cells was extracted using Trizol reagent (Invitrogen, 15596018) and quantified by RT-qPCR using the Roche LightCycler 480 System (Roche Applied Science, Indianapolis, IN, USA). The QuantiTech SYBR Green RT-qPCR kit (Qiagen, 204243) was used to prepare sample mixtures, following a program of 50℃ for 30 min (RT step), 95℃ for 15 min (polymerase activation step), and then 45 cycles at 95℃ for 15 sec, 57℃ for 25 sec, and 72℃ for 10 sec (amplification step). All data was calculated using the $^{-\Delta\Delta}$Ct method, and each experiment was performed in triplicate (Wang et al., 2016).

2.3 $TCID_{50}$ assay

The supernatant from tested cells was collected and subjected to ten-fold serial dilution. The dilution was then added to BHK-21 cells in 96 well plates. Each dilution was repeated 6 times. Following incubation for 4~5 days, CHIKV-induced cytopathic effects (CPEs) were observed by microscope, before cells were fixed and stained using 1% crystal violet solution.

2.4 Immunofluorescence assay (IFA)

The cells were fixed using a mixture of methanol and acetone before human anti-CHIKV antibodies (Dendritics, DDX9100P-50, 1:200) were added

(Selvarajah et al., 2013; Warter et al., 2011) for 1 hour. The cells were then washed using PBS three times and incubated with Alexa Fluor 488-conjugated goat anti-human IgG (Invitrogen, A-11013, 1:500) for 1 hour. At the end of the incubation period, the cells were washed again, and images were captured using an inverted fluorescence microscope (Olympus, IX71).

2.5 Microneutralization assay

U2OS cells were infected with CHIKV S27, 0611aTw, 0810bTw, SINV, or SFV at an MOI of 0.001 in the presence of micafungin at indicated dosages and then incubated for 3 days. The treated cells were then fixed and stained using 0.1% crystal violet solution. EC_{50} values were calculated using GraphPad Prism Version 5, according to the related optical density at 570 nm (OD_{570}) [=($OD_{treatment}$-OD_{CC}) / (OD_{virus}-OD_{CC})], which was measured using a Tecan Infinite 200 Pro multiplate reader. All assays were performed at least three times.

2.6 Cell viability assay

CCK-8 (Sigma-Aldrich, 96992) was used to assess cell viability, in accordance with the manufacturer's protocol. In brief, the U2OS cells were treated with the indicated dosages of micafungin prior to incubation for 3 days. The medium was then replaced with fresh medium that included 10% CCK-8 reagent. After incubation for an additional 1~2 hours, the optical density was measured at 440nm using a Tecan Infinite 200 Pro multiplate reader. All CCK-8 assays were conducted at least three times, and the results were normalized with the control group.

2.7 *In-vivo* toxicity assay

The protocol used in the *in-vivo* toxicity assay of zebrafish was approved by the Institutional Animal Care and Use Committee (IACUC) and conformed to criteria outlined in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. In brief, serial dosages of micafungin were added to embryo medium. Fertilized sphere-stage embryos (4–5 hours post fertilization, hpf) in 24-well plates at a concentration of two embryos/well were then exposed to micafungin solution at the following dosages: 12.5, 25, 50, and 100 μM (equivalent to 15.875, 31.75, 63.5, 127 μg/ml). Each well contained 2 ml of the test solution for 7 days and is updated daily. Following exposure, the embryos were observed daily using a stereomicroscope to evaluate survival, body length, malformation, and hatching. The hatching rate and survival rate were compared with those of the control group. We also recorded morphological anomalies, such as chorion with attached debris, delayed development, lack of spontaneous movement at 1 to 7 dpf, pericardial edema, yolk sac edema, bent trunk, tail malformation, and an uninflated swim bladder. All tested embryos were incubated at 28℃ under a 14 hours light/10 hours dark photoperiod. Following completion of the experiment, all of the zebrafish were sacrificed using 0.5% tricaine (Sigma-Aldrich, MS-222) to minimize suffering.

2.8 Time of addition assay

U2OS cells seeded in 24 well plates were infected with CHIKV S27 at MOI=0.01 for 1h absorption. Following this, the inoculum was replaced with

fresh medium, and cells were incubated for another 16 h. Micafungin (25 μM; 31.75 μg/ml) was either added during the 1h pre-absorption period (pre-treatment), during the absorption period (co-treatment), during the post-absorption period (post-treatment), or during all periods (full-time treatment). Supernatants were later collected for $TCID_{50}$ assays, and the cell lysate was used to extract RNA for RT-qPCR assays.

2.9 Cell-to-cell transmission assay

BHK-21 cells (seeded in a 24 well plate) were infected with CHIKV S27 at an MOI of 0.001 and then incubated for 1 hour. The inoculum was subsequently replaced with fresh medium contained human anti-CHIKV antibodies (neutralization antibodies, Dendritics, DDX9100P-50, 1:100) (Selvarajah et al., 2013; Warter et al., 2011) and the indicated drugs. After incubation for 16 hours, the cells were fixed and stained using J2 anti-dsRNA IgG2a monoclonal antibodies (Scicons, J2-1406, 1:200) as primary antibodies and Alexa Fluor 594-conjugated goat anti-mouse IgG (Invitrogen, R37121, 1:500) as secondary antibodies, in accordance with IFA protocols. DAPI was used for nuclear staining in order to count the number of cells per focus.

2.10 Molecular docking analysis

Docking simulations were performed using docking software, PatchDock, to predict the molecular docking of micafungin and the CHIKV envelope glycoprotein complex (PDB: 3N42). To generate an ensemble of docked conformations, whereupon scoring functions were used to generate classes based on the dock scores (Schneidman-Duhovny et al., 2005).

2.11 Statistical analysis

The Student's t test and Kaplan-Meier test were utilized to analyze data. A p value of <0.05 was considered significant. All statistical analyses were conducted using GraphPad prism software.

3. Results

3.1 Anti-CHIKV activity and safety of micafungin

In this study, we sought to determine the antiviral activity of Micafungin in CHIKV infection. The anti-CHIKV ability of micafungin was first evaluated in U2OS cells, which were infected with CHIKV S27 at MOI=0.01 for 1 hour (absorption) before the inoculum was removed and cells were incubated for another 16 hour (or 24 hour for IFA testing). Serial dilutions of micafungin were added during absorption and after absorption, and results were assessed using RT-qPCR, $TCID_{50}$ assays, and IFA (Fig. 1C and 1D). Micafungin proved effective against CHIKV at the RNA level (Fig. 1A) and the infection level (Figs. 1C and 1D) and was also effective in limiting progeny yield (Fig. 1B). Fig. 1D presents the digital results of IFA, which reveal that micafungin significantly reduced CHIKV infection at concentrations of 25~50 μM (31.75~63.5 μg/ml). However, micafungin does not inhibit progeny yield at low concentrations of 12.5 μM (15.875 μg/ml) which might be due to the result of CHIKV progeny yield with a high standard deviation than RNA level and IFA (Fig. 1B).

Microneutralization assays were also performed to analyze the antiviral ability of micafungin against various strains of CHIKV. The 0611aTw and 0810bTw strains are clinical isolates that were imported from Singapore and Malaysia. Especially, strain 0810bTw with a A226V mutant was related to the

strain that was involved in the outbreak in La Réunion. Micafungin was shown to significantly decrease the cytopathic effects caused by CHIKV S27, 0611aTw, and 0810bTw (Fig. 2A). Micafungin might also allow CHIKV-infected cells to recover to a level similar to that of the control group, particularly when administered at doses of 100 μM (127 μg/ml) and 50 μM (63.5 μg/ml). However, we observed some evidence of cytotoxicity at a concentration of 100 μM (127 μg/ml) (Fig. 2A), and this toxicity was further confirmed by CCK-8 assay (Fig. 2B red line). The OD_{590} value of microneutralization assay was further analyzed using a microplate reader and normalized with the OD_{590} value the cell control to determine survival rates. The IC_{50} values of micafungin were then calculated using GraphPad Prism 6 (Fig. 2B) and were found to be as follows: CHIKV S27 (20.63±1.69 μM; 26.2±2.15 μg/ml), 0611aTw (19.29±1.41 μM; 24.5±1.79 μg/ml), and 0810bTw (17.2±1.08 μM; 21.84±1.37 μg/ml). The CC_{50} values were higher than the 100 μM (127 μg/ml) values. The SI values of micafungin were as follows: CHIKV S27 (>4.85), 0611aTw (>5.18), and 0810bTw (>5.81). *In-vivo* toxicity assays of zebrafish were used to further analyze the toxicity of micafungin. The survival rates between mock groups and groups treated with 100 μM (127 μg/ml) micafungin were not significantly different (Fig. 3A and 3B), indicating that micafungin is safe for *in-vivo* analysis.

3.2 Time of addition assay results

Time of addition assays were used to identify the stages in which micafungin might be effective. For this, U2OS cells were infected with CHIKV S27 at MOI=0.01 for 1 hour absorption. The inoculum was then replaced with fresh medium for 16 hours incubation. Micafungin was either added at a

concentration of 25 μM (31.75 μg/ml) prior to the 1 hour absorption period (pre-treatment), during the absorption period (co-treatment), during the post absorption period (post-treatment), or during all of the periods (full time-treatment) (Fig. 4A). Intracellular RNA was used to verify the ability of micafungin to prevent viral replication. Micafungin (25 μM; 31.75 μg/ml) was found to decrease CHIKV RNA levels by approximately 1 log fold in the post-treatment group and by 4 log fold in the full time-treatment group. Micafungin also observed with a slight affect in CHIKV RNA levels in the pre-treatment group and the co-treatment group (Fig. 4B). $TCID_{50}$ assays were used to verify the extracellular CHIKV progeny yield. In these assays, micafungin was shown to significantly reduce CHIKV progeny generation in the post-treatment (a 3 log fold reduction) and full-time treatment groups (a 7 log fold reduction). Micafungin did not affect CHIKV progeny levels in the pre-treatment or co-treatment groups (Fig. 4C). Micafungin proved to be more effective in inhibiting CHIKV progeny yield than in reducing RNA replication (Fig. 4C). These results indicate that micafungin has other effects following virus RNA replication and therefore suggests that micafungin could have a major influence on the later stages of CHIKV infection. However, the inhibitory effects of micafungin were stronger in the full-time treatment group than in the post-treatment group. This finding suggests that multiple mechanisms may be responsible for the beneficial effects that micafungin has on CHIKV infection.

3.3 Micafungin combats CHIKV infection by limiting RNA replication, virus release, virion stability, and cell-to-cell transmission

We further assessed the inhibitory effects of micafungin following CHIKV

infection. For this, U2OS cell were infected with CHIKV at MOI=0.01 for 1 hour (absorption), the inoculum was removed, and micafungin was added at the indicated dosages for a 16 hours incubation period. Cell lysate and supernatant were collected for RT-qPCR and $TCID_{50}$ assays to verify CHIKV RNA replication levels and CHIKV release levels. We found that CHIKV RNA levels decreased by approximately 1.59 log fold at a dose of 50 μM (63.5 μg/ml) and 0.5 log fold at a dose of 25 μM (31.75 μg/ml) (Fig. 5A). Furthermore, the release of CHIKV virions dropped by approximately 3.99 log fold at a dose of 50 μM (63.5 μg/ml) and by 1.46 log fold at a dose of 25 μM (31.75 μg/ml) (Fig. 5B). We also assessed whether micafungin affects CHIKV stability. For this, micafungin was added to the CHIKV supernatant at indicated concentrations and the mixture was then held in an incubator at 37℃ for 16 hours. Micafungin was only able to inhibit CHIKV stability at a concentration of 50 μM (63.5 μg/ml) (Fig. 5C, a reduction of approximately 0.6 log fold). Besides, micafungin could not inhibit CHIKV binding and entry (shown as Supplementary Fig. 1). Overall, our results demonstrate that micafungin inhibits CHIKV RNA replication and CHIKV release and has at least some effect on CHIKV stability.

Cell-to-cell transmission is another pathway related to CHIKV transmission. In a previous study, we found that suramin may inhibit cell-to-cell transmission in BHK cells infected with CHIKV. We therefore used suramin as a positive control. Our results revealed that both micafungin (25 μM; 31.75 μg/ml) and suramin (175 μM) significantly inhibited the cell-to-cell transmission of CHIKV. Fig. 5D presents the cell count per focus.

3.4 Micafungin may possess broad spectrum anti-alphavirus ability

We also evaluated the inhibitory effects of micafungin against other alphaviruses: SINV and SFV. Microneutralization assays, RNA levels, and CHIKV progeny yields all confirmed the inhibitory effects of micafungin (Fig. 6A~E). Specifically, micafungin was shown to reduce virus propagation at concentrations of 50 μM (63.5 μg/ml) and 25 μM (31.75 μg/ml). Micafungin also presented inhibitory effects at a concentration of 100 μM (127 μg/ml), albeit with slight toxicity (Fig. 6A). At a concentration of 12.5 μM (15.875 μg/ml), micafungin revealed a little high level compared to SFV infected group (Fig. 6C and 6E). Overall, these results indicate that micafungin may possess broad spectrum effects against alphavirus infection.

3.5 Docking analysis of micafungin with the CHIKV envelope glycoprotein complex

We employed the docking software, PatchDock, to predict the molecular docking of micafungin and the CHIKV envelope glycoprotein complex. The highest Patchdock score (9722) was obtained for the model in which suramin embedded within the cavity of the CHIKV envelope glycoprotein complex between E1 domain II and E2 domain A (Supplementary Fig. 2). The evidences imply that molecular docking results revealed that micafungin is able to bind with CHIKV envelope proteins.

4. Discussion

Micafungin was first reported to counter the proliferation of EV71 and the replication of EV71 replicons in cells at an IC_{50} value of 5 μM (6.35 μg/ml) (Kim et al., 2016). CVB3 is a member of human enterovirus B group (HEV-B) and is

one of the main causes of viral meningitis, myocarditis, and pancreatitis (Sawyer, 2002; Whitton et al., 2005). HRV belongs to the genus Enterovirus (along with EV71 and CVB3) and is the cause of the common cold (Kang et al., 2015). Thus, both are important concerns for public health (Kim et al., 2016). The fact that micafungin possesses moderate antiviral activity against both enteroviruses (Kim et al., 2016) suggests that it may possess broad spectrum anti-enterovirus properties.

In this study, we first demonstrated the inhibitory effects of micafungin against chikungunya virus infection. The fact that micafungin affected two clinical strains of chikungunya as well as SINV and SFV, indicates that this antifungal agent may provide broad spectrum inhibitory effects against alphavirus infections. Micafungin presented slight cytotoxicity at a concentration of 100 μM (127 μg/ml); however, the CC_{50} of micafungin was >100 μM (127 μg/ml). In the preclinical studies, results showed the development of benign liver tumors in rats treated with extremely high doses of the micafungin for prolonged periods (Valerio et al., 2011). To date, no relevant liver or renal dysfunction has been found in this largest patient cohort, and intermittent administration of high doses of micafungin was well tolerated (Neofytos et al., 2015). In *in-vivo* zebrafish toxicity assays, micafungin did not show any indications of toxicity in terms of survival rate, hatching rate, or morphology. A retrospective, observational, exploratory cohort study clarified the safety of micafungin at a daily dose of more than 150 mg. Twenty-six patients administered micafungin at 300 mg daily (high-dose group) were compared with 58 patients administered micafungin at 150 mg daily (standard-dose group) for $\geq$ 7 consecutive days. Serious adverse events (AEs)

and resultant treatment discontinuation were infrequent. These results suggest that micafungin was safe and well tolerated at 300 mg daily (Yamazaki et al., 2014). The common side effects of micafungin are including nausea, vomiting, diarrhea, fever, headache and thrombocytopenia. Even though the dose-limiting toxicity was reported up to a daily dose of 8 mg/kg (896 mg), one patient was reported without any side effect when administered 1400 mg micafungin every two weeks for 12 weeks. The effective concentrations of micafungin ranged from 12.5 to 100 μM (15.875 to 127 μg/ml). The IC_{50} of micafungin against CHIKV S27 and the two isolates ranged from 17.2 to 20.63 μM (20.63 to 26.2 μg/ml), which was higher than the IC_{50} value against EV71 (5 μM (6.35 μg/ml)). As above discussion, when patient received 150 mg daily dose, the C_{max} values were about 12~16 mg/L (12~16 μg/ml). The concentration of 50μM (approximately 60μg/ml) was about 4-fold higher than typical micafungin exposure but was still under the limiting dose (896 mg). Therefore, the concentration of 50 μM of micafungin might be safety to use in clinical. This may be due to the rapid disease progression of CHIKV or the methods used in the analysis of infection. In time of addition assays, micafungin also showed inhibitory effects in later stages of CHIKV infection. The full-time treatment group presented a more significant decrease than did the post-treatment group. The effects of micafungin were more pronounced on CHIKV progeny yield than on CHIKV RNA. This evidence suggests that multiple mechanisms are involving in the inhibitory effects of micafungin against CHIKV infection.

To elucidate the effects of micafungin in the later stages of CHIKV infection, we focused on the period after CHIKV entry into cells. Micafungin

was shown to inhibit CHIKV RNA production and virus release and also to reduce virion stability. In subsequent analysis, we found that a micafungin concentration of 50 uM led to a remarkable reduction in virus progeny yield (3.99 log fold), and this reduction far exceeded that of RNA yield (1.59 log fold) and stability (0.6 log fold). Similar results were obtained at a micafungin concentration of 25 uM; however, this concentration was insufficient to affect virion stability. These findings clearly show that micafungin inhibits virus release and reduces the production of viral RNA. The findings also reveal that virion stability is only influenced at higher dosages of micafungin. Micafungin was also shown to affect the cell-to-cell transmission of CHIKV. In a previous study, we determined that those compounds could bind with CHIKV envelope proteins, thereby affecting the entry, release, and cell-to-cell transmission of the virus. Molecular docking results revealed that micafungin is able to bind with CHIKV envelope proteins (Supplementary Fig. 2); however, micafungin did not significantly inhibit viral progeny yield during the early stages of CHIKV infection (Fig. 4C). Following virus entry into the cell, envelope proteins must undergo conformational changes to enable the release of viral RNA. We suspect that the binding site of micafungin may not prevent conformational changes in envelope proteins, which could explain why micafungin had no effect on CHIKV entry, but did influence virus release and cell-to-cell transmission.

Micafungin is a non-reversible inhibitor which inhibits the synthesis of the cell wall by targeting β-1,3-D-glucan synthase of fungi (Douglas, 2001). Although the fungus is a eukaryote, such as a human, the cell wall is not shared by mammalian cells or viruses (Denning, 2003). In this respect, the

antiviral effect of micafungin seems to have nothing to do with inhibition of β-1,3-Dglucan synthase. Unfortunately, more biological activity or cellular targets of micafungin has not been defined, so it is not easy to explain the extra mechanism of antiviral effects on CHIKV. However, we could speculate that micafungin may target patterns of intracellular events during CHIKV infection, such as viral replication, intracellular and extracellular transmission and virus stability. Similar results also appeared in previous EV71 studies (Kim et al., 2016). The molecular docking was used to predict the interaction between micafungin and CHIKV envelope proteins which might help to explain how micafungin suppress CHIKV release, cell-to-cell transmission and stability. However, we did not have any evidence to support whether micafungin against CHIKV replication through inhibition of viral replicase or affect the host target to against CHIKV replication.

In our future work, we might use RNA sequencing to analyze which genes will be regulate during micafungin administration and figure out which genes might contribute to viral replication. In this study, our findings suggested that micafungin were able to inhibit CHIKV replication, transmission, and virus stability which could provide a basis for the development of novel human drug therapies against life-threatening CHIKV and other alphavirus infections.

Author contributions

YJH and JWL conceived and designed the experiments. YJH, FCL, CTY, CMY and JWL performed the experiments. YJH and JWL analyzed the data. YJH, CCL, TYL, PSH, ZG, and JWL contributed reagents, materials, and analytical tools. YJH and JWL wrote the paper. YJH, FCL, CCL, POH, MKH, ZG, and

LJW helped interpret results.

Conflict of interest

None.

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Figure legends

Fig. 1. Dose-dependent anti-CHIKV activity of Micafungin. (A) RT-qPCR assay. (B) $TCID_{50}$ assay. (C) IFA. (D) Quantification of the percentage of infected cells of IFA.

Fig. 2. IC_{50} and CC_{50} of Micafungin. (A) Microneutralization assay. The protective abilities of micafungin against infection with CHIKV S27, 0611aTw, and 0810bTw was verified using microneutralization assays. Mock refers to cell control group samples which were not infected with any virus. (B) Quantification of microneutralization assays and CCK-8 assays. In the figure, values obtained using microneutralization assays are represented as follows: CHIKV S27 (blue line), 0611aTw (green line), and 0810bTw (black line). The quantification of cell viability was using CCK-8 assays (red line). The table below lists the IC_{50}, CC_{50}, and SI values.

Fig. 3. *In-vivo* toxicity assays in zebrafish. (A) The survival rate of larvae fish exposed to micafungin 100 to 12.5 μM (127 to 15.875 μg/ml) for 7 days was determined using a Kaplan-Meier plot (n=70 fish per treatment group). (B) Photo of treated larvae fish on day 7.

Fig. 4. Time of addition assay. (A) Timeline of time of addition assays. The red line refers to the CHIKV absorption period and blue line refers to the micafungin administration period. (B) RT-qPCR was used to assess intracellular CHIKV RNA levels. (C) $TCID_{50}$ assays were used to assess extracellular CHIKV progeny yield.

Fig. 5. Effects of micafungin following CHIKV entry into cells. (A) Fold change of CHIKV RNA replication under micafungin treatment. (B) Fold change of CHIKV release under micafungin treatment. (C) Fold change of CHIKV stability under micafungin treatment. (D) Cell-to-cell transmission levels of CHIKV under micafungin treatment.

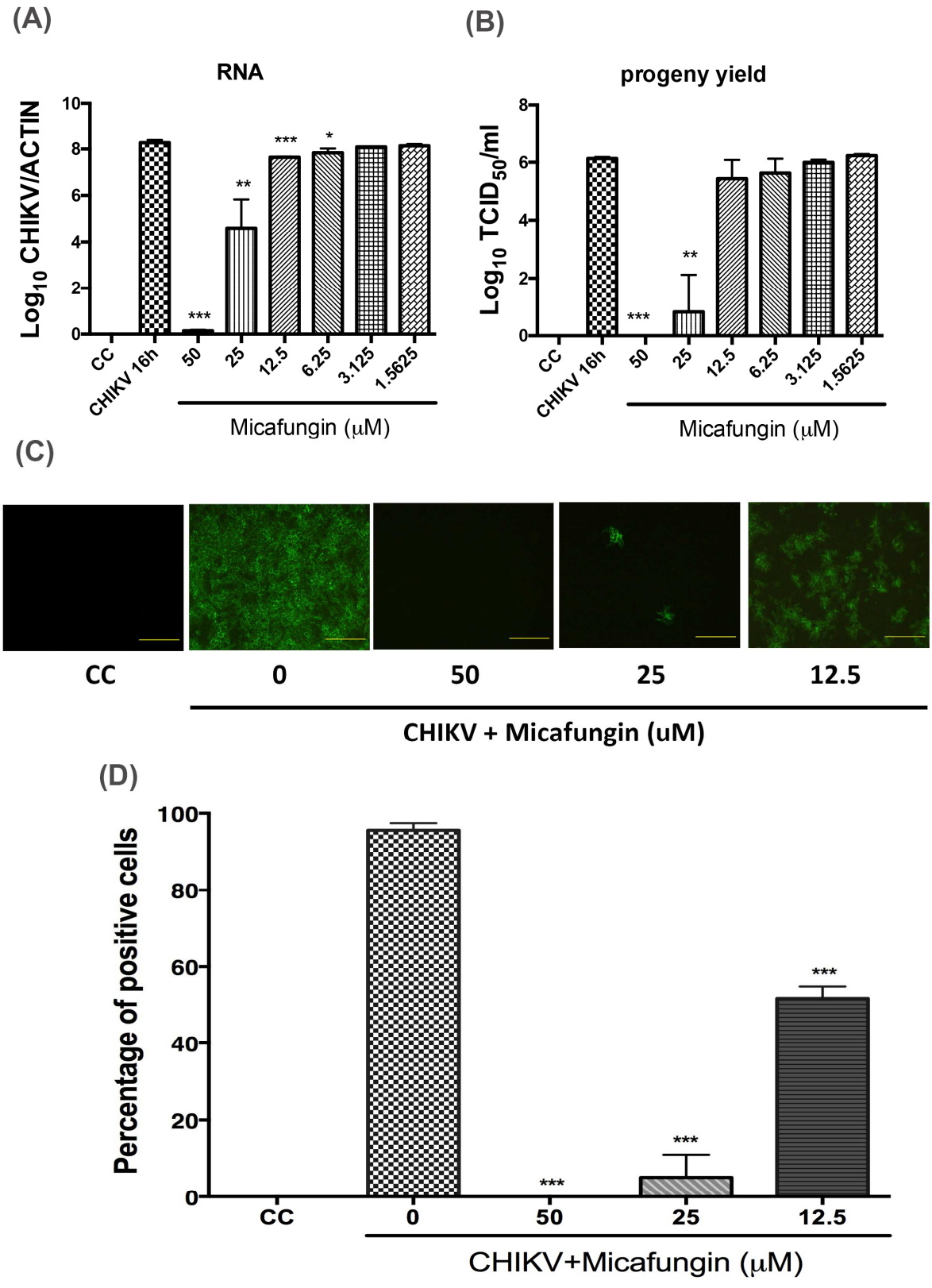
Fig. 6. Antiviral effects of micafungin against SINV and SFV infection. (A) microneutralization assays. (B and C) RNA levels of SINV and SFV under micafungin treatment. (D and E) Viral progeny yield of SINV and SFV under micafungin treatment.

Supplementary Figure legends

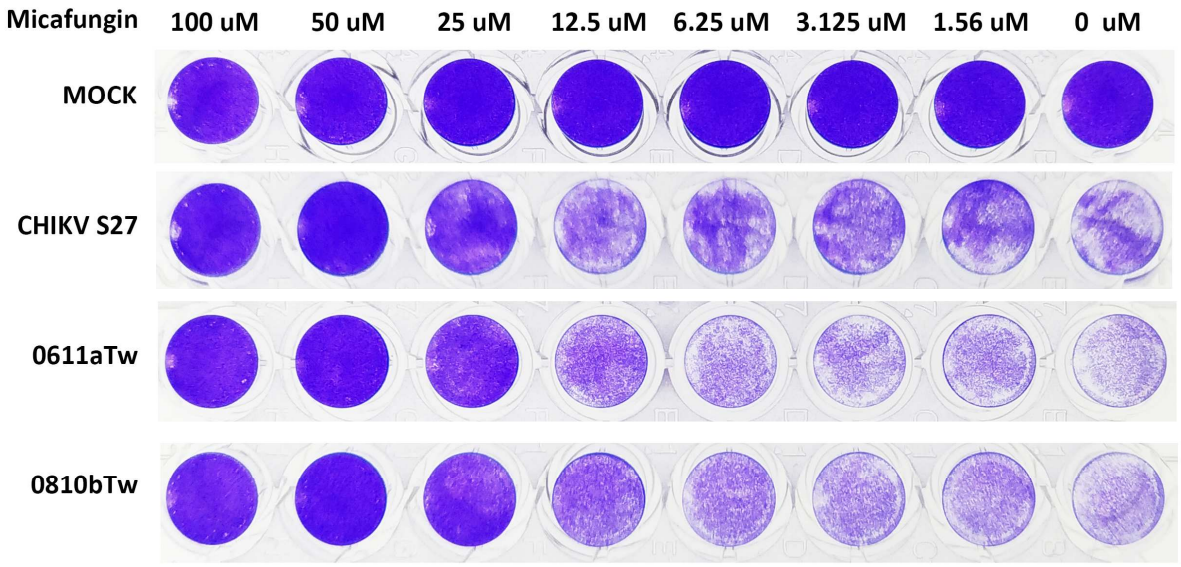
Supplementary Fig. 1. The effect of micafungin at the early stage of CHIKV infection. (A) The binding assay. U2OS cell were incubated with indicated dosage of micafungin and CHIKV at MOI=1 at 4℃ for 1 hour (absorption) and then the inoculum was removed. Micafungin was added at the indicated dosages for a 5 hours incubation period and the RNA was detected by RT-qPCR. (B) The entry assay. U2OS cell were incubated with indicated dosage of micafungin and CHIKV at MOI=1 at 37℃ for 1 hour (absorption) and then the inoculum was removed. Micafungin was added at the indicated dosages for a 7 hours incubation period and the RNA was detected by RT-qPCR.

Supplementary Fig. 2. The molecular docking of micafungin and CHIKV envelope proteins (PDB:3N42). E1 with domain I, II, III labeled in pink, E2

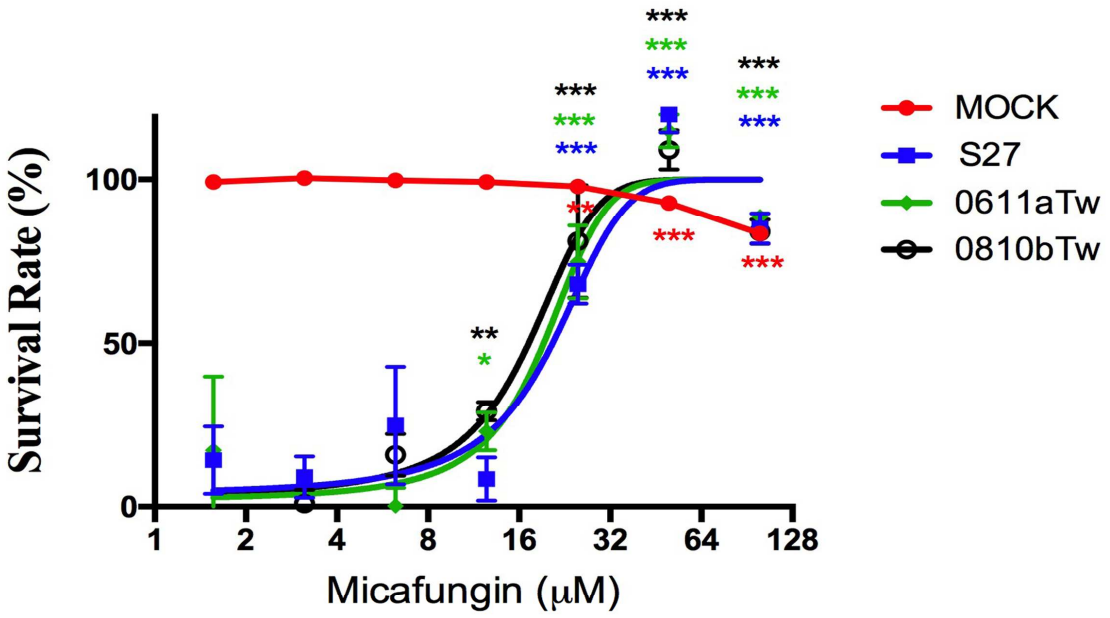
with domain A, B, C and b-ribbon labeled in blue, E3 labeled in green. Micafungin was bound in the cavity of E1 and E2.


(A)
RNA
Log10 CHIKV/ACTIN
10
8
6
4
2
0
***
**
***
*
CC
CHIKV 16h
50
25
12.5
6.25
3.125
1.5625
Micafungin (μM)
(B)
progeny yield
Log10 TCID50/ml
8
6
4
2
0
***
**
CC
CHIKV 16h
50
25
12.5
6.25
3.125
1.5625
Micafungin (μM)
(C)
CC
0
50
25
12.5
CHIKV + Micafungin (uM)
(D)
Percentage of positive cells
100
80
60
40
20
0
***
***
***
CC
0
50
25
12.5
CHIKV+Micafungin (μM)

(A)

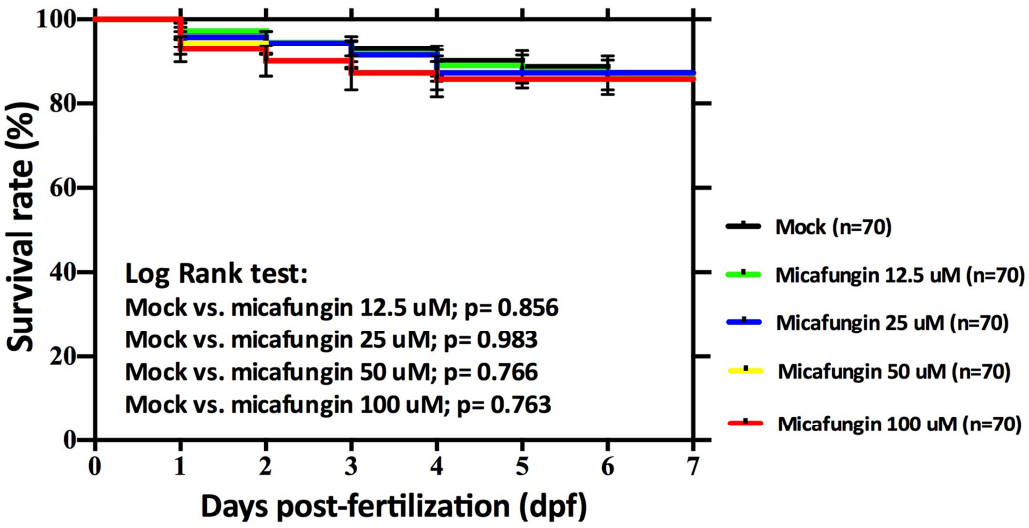


(B)



Strains	IC_{50}(μM)	CC_{50}(μM)	SI
CHIKV S27	20.63±1.69	>100	>4.85
0611aTw	19.29±1.41	>100	>5.18
0810bTw	17.2±1.08	>100	>5.81

(A)



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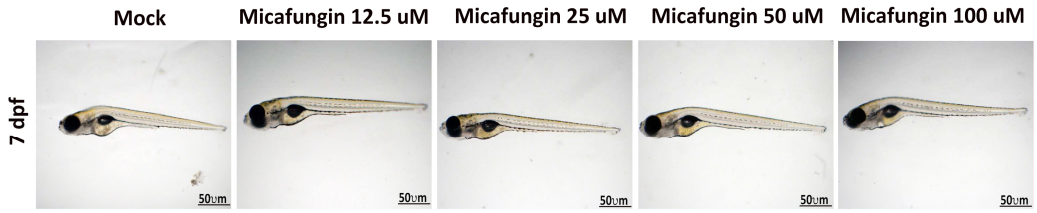

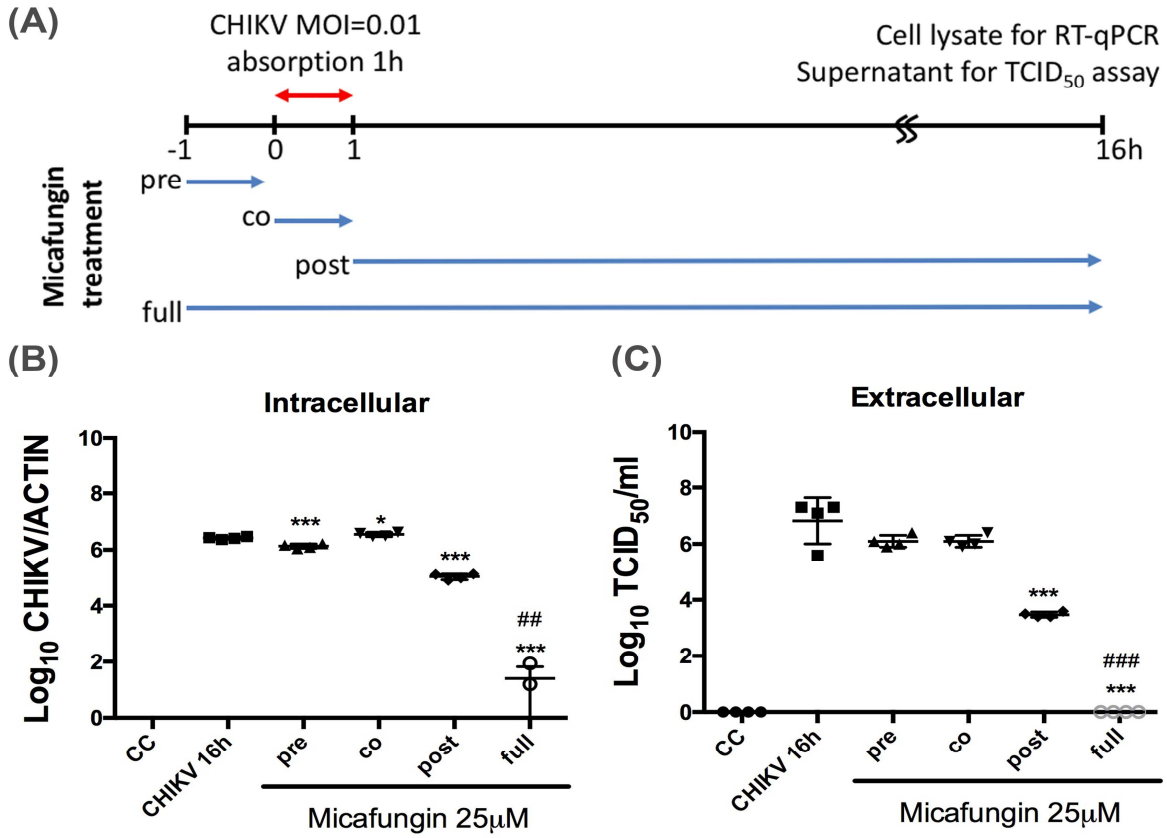

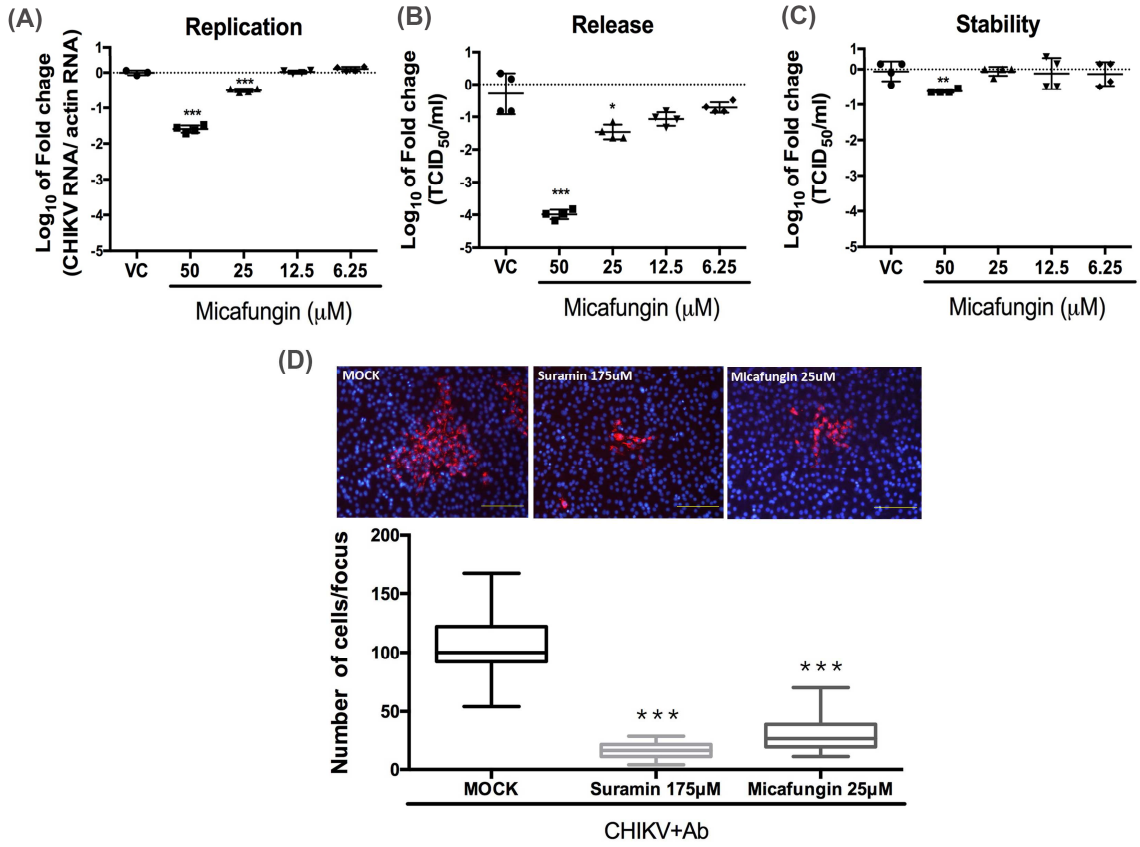

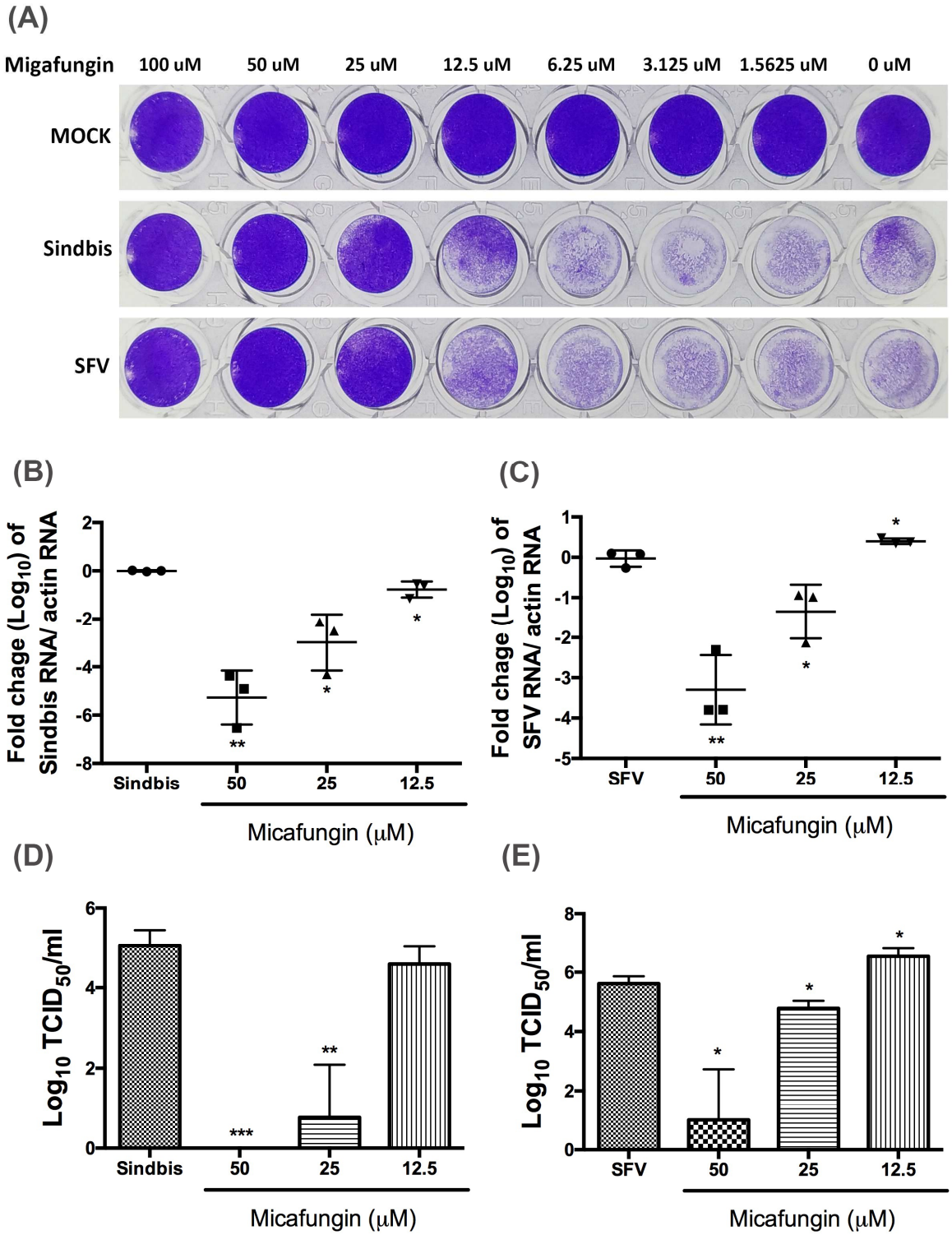
(A)
Migafungin
100 uM
50 uM
25 uM
12.5 uM
6.25 uM
3.125 uM
1.5625 uM
0 uM
MOCK
Sindbis
SFV
(B)
Fold chage (Log10) of Sindbis RNA/ actin RNA
Sindbis
50
25
12.5
Micafungin (μM)
(C)
Fold chage (Log10) of SFV RNA/ actin RNA
SFV
50
25
12.5
Micafungin (μM)
(D)
Log10 TCID50/ml
Sindbis
50
25
12.5
Micafungin (μM)
(E)
Log10 TCID50/ml
SFV
50
25
12.5
Micafungin (μM)

Highlights

•Micafungin was identified as a new anti-CHIKV agent.

•The anti-CHIKV effect of micafungin is also proved by CHIKV two clinical isolates.

•Micafungin could also possess the ability to against other alphaviruses.