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ORIGINAL ARTICLE

A Selective Histone Deacetylase 6 Inhibitor Showed Antiviral Activity Against Dengue and Zika Viruses

Hao-Wen Shih[1], Chang-Huei Tsao[1,2], Yu-Hsiu Chang[3], Chih-Wei Yang[4], Shao-Wei Feng[5], Kuo-Chou Chiu[6,7]

[1]Department of Microbiology and Immunology, National Defense Medical Center, [2]Department of Medical Research, Tri-Service General Hospital, National Defense Medical Center, [3]Institute of Preventive Medicine, National Defense Medical Center, [4]Division of Gastroenterology, Department of Internal Medicine, Tri-Service General Hospital, National Defense Medical Center, [5]Department of Neurological Surgery, Tri-Service General Hospital, National Defense Medical Center, [6]Department of Family Dentistry, Tri-Service General Hospital, National Defense Medical Center, [7]School of Dentistry, National Defense Medical Center, Taipei, Taiwan

Background: Flavivirus comprises several important viruses, including dengue virus (DENV), Zika virus (ZIKV), and Japanese encephalitis virus (JEV). A large outbreak of DENV and ZIKV occurred in these years, leading to many cases of illness and death. However, despite the decades of efforts, there are no specific therapeutic drugs against DENV and ZIKV. Several studies had shown that histone deacetylase 6 inhibitors (HDAC6 inhibitors) possess antiviral effects on influenza A virus, hepatitis C virus, and JEV. **Aim:** The purpose of this study is to examine the antiviral effect of the compound J34803, a newly synthesized HDAC6 inhibitor, against DENV and ZIKV *in vitro* and *in vivo*. **Methods:** We investigated whether the compound J34803 inhibited viral infection by western blot and virus titer determination. The signaling pathway of inhibition was also determined by western blot. **Results:** The compound J34803 exhibited superior antiviral activities against DENV-2, DENV-4, and ZIKV compared to Tubastatin A (TBSA), and its antiviral mechanism may through suppressing HDAC6 and its downstream signaling pathway. Moreover, treatment with the compound J34803 could reduce viremia levels in DENV-2-and ZIKV-infected AG129 mice. **Conclusion:** We demonstrated that the compound J34803 had better therapeutic efficacy in virus infection as compared to TBSA and could be a potential potent therapeutic drug against emerging flaviviral infections.

Key words: Flavivirus, dengue virus, Zika virus, anti-viral drugs, histone deacetylase 6 inhibitors

INTRODUCTION

Flaviviruses, viruses with single-stranded and positive sense RNA, have three structural proteins and seven nonstructural proteins. Capsid (C), precursor membrane/membrane (prM/M), envelope (E) protein and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) are formed by cleaving a single polyprotein which is encoding from the viral RNA with host and viral protease.[1] The Flavivirus genus of viruses contains dengue (DENV), Zika (ZIKV), yellow fever, Japanese encephalitis (JEV), and West Nile viruses.[2] Among the mosquito-borne flaviviruses, there are four serotypes of DENV that cause infection and can lead to illnesses ranging from dengue fever and severe dengue hemorrhagic fever (DHF) to dengue shock syndrome (DSS).[3-5] Besides, ZIKV infection may cause severe neurological complications in adults such as Guillain-Barré syndrome or microcephaly, congenital malformation, and fetal demise in fetuses.[6-8] However, there are still no specific antiviral drugs approved for the treatment of DENV and ZIKV infection to date. Thus, the development of anti-flaviviral drugs is crucially needed to against these severe and fatal diseases.

Histone deacetylases (HDACs) are a family of enzymes that function in gene expression, chromatin remodeling, protein

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Corresponding Author: Dr. Kuo-Chou Chiu, Department of Family Dentistry, Tri-Service General Hospital, No. 325. Sec. 2, Chenggong Rd., Neihu Dist., Taipei 114, Taiwan. Tel: +886287923311 ext. 13823; Fax: +886297927147. E-mail: scalingdentist61@yahoo.com.tw

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stability, and transport.[9,10] HDAC6 is a unique deacetylase enzyme in the HDAC family. It possesses two deacetylase domains and a zinc finger motif (ubiquitin binding), mostly localizes in the cytoplasm and mediates various cellular processes by deacetylating nonhistone substrates, including heat shock protein 90 (HSP90), α-tubulin, and cortactin.[11,12] HDAC6 also interacts with polyubiquitinated or misfolded proteins, leading to aggresome formation and protein degradation.[13]

HDAC6 interacts with proteins involved in cell migration, immune response, transcription, cell proliferation and death, the degradation of misfolded proteins, and stress response pathways.[14] In addition, several studies have indicated that HDAC6 plays an important role in the regulation of apoptosis. Ku70 binds to Bax, inhibiting Bax-induced cell death.[15] Cytoplasmic Ku70 acetylation status is regulated by HDAC6.[16] Deacetylated Ku70 stays in a complex with Bax, whereas acetylated Ku70 releases Bax, triggering apoptosis in neuroblastoma cells.[14] Moreover, HDAC6 is implicated in virus infection. For example, HIV-1 fusion and infection are inhibited by HDAC6-mediated deacetylation of α-tubulin.[17] Influenza A virus can utilize the aggresome-processing machinery mediated by HDAC6 to facilitate its uncoating and infection.[13] Currently, most HDAC inhibitors used in the clinic are nonselective HDAC inhibitors. HDAC inhibitors exhibit therapeutic potential to viral infection.[18-23] Pan-HDAC inhibitors such as trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA), and valproic acid block the zinc-containing catalytic domain of HDACs.[18,19] TSA reduces the number of viral genomes in Herpes Simplex Virus-1 infected cells.[20] SAHA activates HIV-1 from latency period.[24] Unfortunately, several HDAC inhibitors have been discover undesirable side effects, including Vorinostat (hereafter referred to as SAHA) and Panobinostat, due to their nonselective properties.[25] Meanwhile, there are several papers show selective HDAC6 inhibitors have anti-flavivirus ability. For instance, Tubacin treatment caused the decrease of the Hsp90-NS5 interaction and the reduction of viral proteins and antisense RNA genomes in infected cells.[24] Another example, Tubastatin A (TBSA), an HDAC6-selective inhibitor, decreased in viral RNA concentration in hepatocyte cell infected with hepatitis C virus (HCV) replicon.[26]

However, HDAC6 inhibitors have not been proven to have the ability to against dengue and Zika infection. In this study, we used a newly synthesized selective HDAC6 inhibitor, compound the compound the compound J34803, to evaluate the antiviral effects of compound the compound the compound J34803 against DENV and ZIKV infection *in vitro* and *in vivo*. We demonstrated the superior therapeutic efficacy of the compound J34803 (compared to TBSA), identifying that HDAC6 activities are involved in DENV and ZIKV replication. In this study, we provided a potential potent therapeutic drug against emerging flaviviral infections.

MATERIALS AND METHODS

Ethical approval

The animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the National Defense Medical Center (permit no. IACUC-19-320).

Cell lines and viruses

The baby hamster kidney cell line BHK-21 (ATCC CCL-10) and the *Aedes albopictus* C3/36 cell line (ATCC CRL-1660) were cultured in RPMI-1640 (GIBCO) medium containing 5% Fetal bovine serum (FBS; GIBCO). The human embryonic kidney cell line HEK-293 (ATCC CRL-1573) was cultured in Dulbecco modified Eagle medium (DMEM; GIBCO) containing 10% FBS. The African green monkey kidney cell line Vero (ATCC CCL-81) was cultured in minimal essential medium (MEM; Hyclone) containing 10% FBS.

Two serotypes of DENV (DENV-2, 16,681 strain and DENV-4, H241 strain) were amplified in C6/36 cell line, and viral titers were measured by focus-forming assay in BHK-21 cells. The ZIKV PRVABC59 strain was amplified in C6/36 cell line, and viral titers were measured by focus-forming assay in Vero cells.

Drug cytotoxicity assay

HEK-293 cells were treated with the compound J34803 at the indicated doses for 48 h and analyzed by using the cell proliferation reagent WST-1. Sample absorbance from WST-1 was measured by using an ELISA (enzyme-linked immunosorbent assay) reader at 450 nm.

Western blotting

To investigate the antiviral effects of the compound J34803, HEK-293 cells were infected with DENV-2, DENV-4 or ZIKV, with or without compound treatment. After infection for 24 h, the cell lysates were analyzed by Western blot with the antibodies anti-DENV NS3 protein (GTX2811; GeneTex), anti-ERK1/2 (9102; cell signaling), anti-ERK1/2 phospho Thr202/Tyr204 (9101; cell signaling), anti-Caspase 3 (9662; cell signaling), anti-PARP (9542; cell signaling), anti-HDAC6 (7558; cell signaling), and anti-GAPDH (MAB374; Millipore). Then, the membranes were probed with horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit IgG secondary antibodies (31430 and 31460; Thermo). The signals were developed by enhanced chemiluminescence and photographed by a Luminescent Image Analyzer (LAS-3000; Fujifilm).

Mouse model

The animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the National Defense Medical Center (permit no. IACUC-19–320). To examine the therapeutic efficacy of the compound J34803 against DENV-2 or ZIKV *in vivo*, AG129 mice were divided into two groups for treatment: (1) intraperitoneal (i.p.) injection with 100 PFU/ml of DENV-2 or 10 PFU of ZIKV and PBS (vehicle control); (2) i.p. injection with 100 PFU/ml of DENV-2 or 10 PFU/ml of ZIKV and then i.p. with 5 mg/kg of the compound J34803 every day for 10 days. The survival rates of the mice were monitored daily for 30 days. For viremia detection, serum samples were collected on the 3 day of postinfection. Viral titers were measured using a focus-forming assay or plaque forming assay.

Statistical analysis

GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA) was used for the data analysis of virus titers and viremia levels. The data were analyzed using the Student's *t*-test (*present as statistically significant, $*P < 0.05$; $**P < 0.01$). Survival curves were analyzed by a log-rank test. Bands of Western blot were quantified by using ImageJ software (National Institutes of Health, Bethesda, Maryland, U.S.A.).

RESULTS

The compound J34803 has no cytotoxic effect in HEK-293 cells

Since TBSA is a potent and selective HDAC6 inhibitor, the compound J34803 was synthesized based on the structure of TBSA and retained the active structural domain[27] [Figure 1a]. First, we examined the cytotoxic effect of the compound J34803 in HEK-293 cells using the WST-1 cell proliferation assay. We chose HEK-293 as our cell line due to HEK-293 has been chosen for flavivirus infection in several studies.[28] To verify whether the compound J34803 is toxic in HEK-293 cells, we treated HEK-293 with different concentrations of the compound J34803 for 48 h. Treatment with the compound J34803 did not demonstrate cytotoxicity at concentrations up to 15 μM in HEK-293 cells for 48 h [Figure 1b].

The compound J34803 exhibited superior antiviral activities against dengue virus-2, dengue virus-4, and Zika virus rather than Tubastatin A

It has been reported that TBSA could inhibit the proliferation of the JEV and HCV.[24,29] Thus, we try to investigate the effect of the compound J34803 against DENV and ZIKV compared to TBSA. HEK-293 cells were infected with DENV-2, DENV-4, or ZIKV (MOI = 1) for 2 h and treated with 5 μM, 10 μM, or 15 μM for 24 h based on the drug concentrations test in

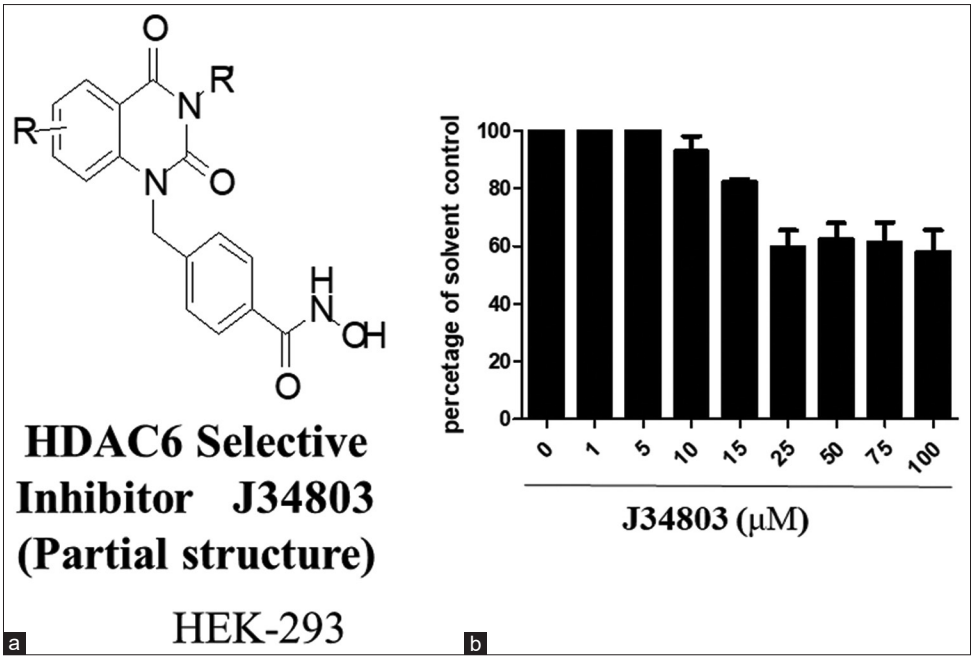

Figure 1: Molecular structure and drug cytotoxicity of the compound J34803. (a) Schematic structure of the compound J34803. (b) HEK-293 cells were treated with solvent or with different concentrations of the compound J34803 for 48 h. The cell viabilities were measured by WST-1 assay. A percentage was obtained by the comparison with the solvent control, set at 100%. Data are the mean ± SD of two independent experiments. According to two-tailed Student's *t*-test, $*P < 0.05$, $**P < 0.01$. SD: Standard deviation

the Figure 1. As shown in Figure 2, the compound J34803 significantly inhibited viral protein expression [Figure 2a] and viral titers [Figure 2b] in a dose-dependent manner in comparison with TBSA [Figure 2c and d]. Overall, the compound J34803 showed superior therapeutic efficacy against DENV-2, DENV-4, and ZIKV.

The compound J34803 inhibited dengue virus-2 and Zika virus replication through suppressing histone deacetylase 6 and its downstream signaling pathway

Due to the compound J34803 could inhibit HDAC6 and virus replication, we further investigated the anti-viral mechanism of the compound J34803 and determined whether the compound J34803 inhibited DENV and ZIKV by suppressing HDAC6 and its downstream signaling pathway, such as ERK,[30,31] Caspase 3, PARP, and ultimately leading cells to apoptosis.[32-34] HEK-293 cells were infected with DENV-2 for 2 h (MOI = 1) and treated with or without 5, 10, or 15 μM of the compound J34803. As shown in Figure 3a and b, infection with DENV-2 or ZIKV in HEK-293 cells stimulated the levels of cleaved Caspase 3 and cleaved PARP (Panel A and B, lane 2) compared to mock-infected HEK-293 cells (Panel A and B, lane 1). Furthermore, treatment with the compound J34803 inhibited HDAC6 and significantly increased the levels of phosphorylated

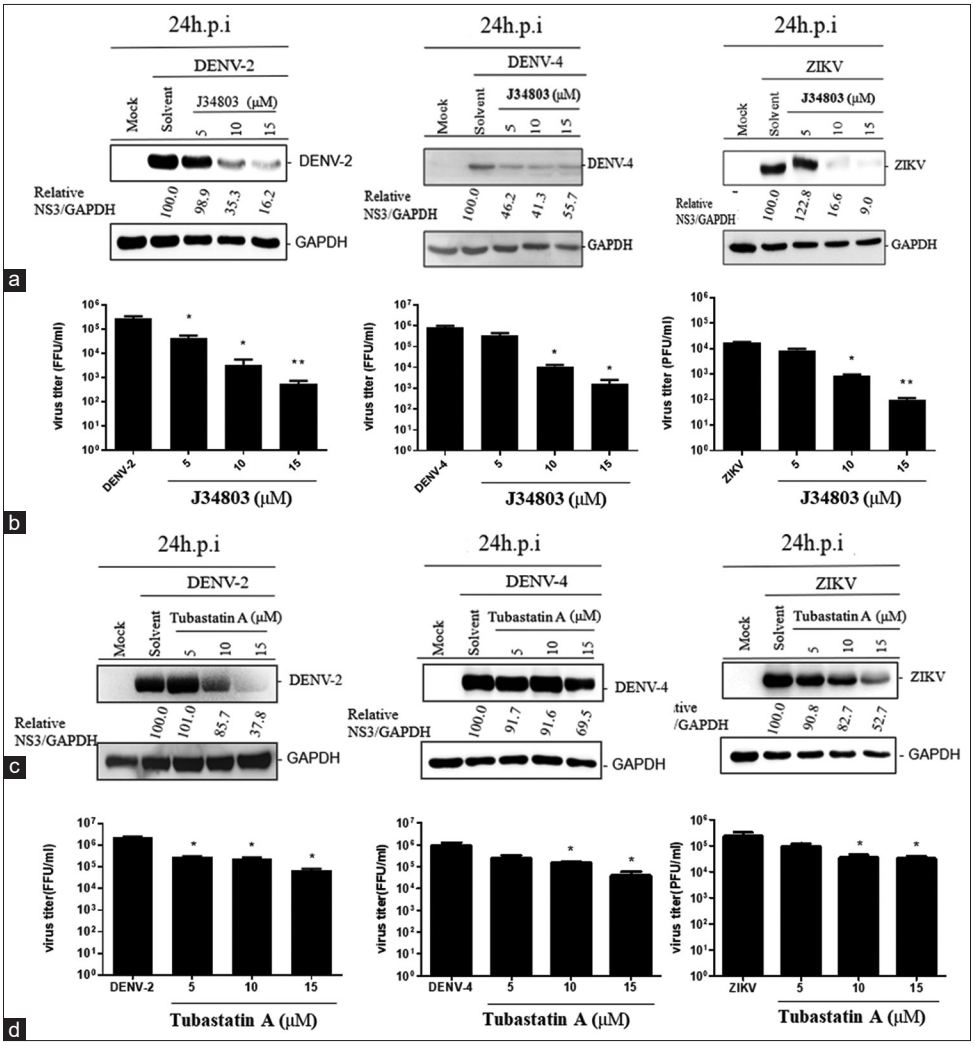

Figure 2: Antiviral activities of the compound J34803 against DENV and ZIKV in HEK-293 cells compared to TBSA. HEK-293 cells were infected with DENV-2, DENV-4, and ZIKV (MOI = 1) without (solvent) or with various concentrations of the compound J34803 and TBSA for 24 h. Viral protein expression levels (a and c) were determined by Western blot analysis. GAPDH was used as a loading control. Relative ratios of viral NS3 protein expression levels to GAPDH levels were adjusted to those of the solvent control. Virus titers (b and d) were analyzed and adjusted to those of the solvent control. Data are the mean ± SD of two independent experiments. According to two-tailed Student's *t*-test, $*P < 0.05$, $**P < 0.01$. TBSA: Tubastatin A, DENV: Dengue virus, ZIKV: Zika virus

ERK, cleave Caspase 3, and cleaved PARP, inhibiting the viral protein expression (Panel A and B, lanes 3–5) in a dose-dependent manner. Thus, the results showed that the compound J34803 do reduce the level of HDAC6 and inhibit viral protein expression by apoptosis through the pathway of ERK, Caspase 3, and PARP in DENV-2 - or ZIKV-infected HEK-293 cells.

The compound J34803 reduced viremia levels in dengue virus-2-and Zika virus-infected AG129 mice

It has been reported that AG129 mice are a suitable animal model for DENV and ZIKV infection.[35,36] To examine the therapeutic efficacy of the compound J34803 against DENV-2 and ZIKV *in vivo*, we infected with DE

NV-2 or ZIKV in AG129 mice and intraperitoneally treated with the compound J34803 at 5 mg/kg once daily for 10 days. Next, we monitored the survival rates and measured viremia levels of DENV-2 or ZIKV in serum from AG129 mice treated with the compound J34803 on day 3 postinfection. As shown in Figure 4a and b, the compound J34803 significantly decreased viremia levels in DENV-2-and ZIKV-infected AG129 mice. However, the survival rates of these mice treated with the compound J34803 were all dead (data not shown).

DISCUSSION

DENV and ZIKV, mosquito-borne flaviviruses, cause various severe illness such as DHF, DSS, and severe neurological complications, respectively.[4,5,7] Moreover, DENV and ZIKV become a threat of public health due to millions of infections annually.[37] Previous study had shown that selective HDAC6 inhibitors have anti-JEV ability. However, HDAC6 inhibitors have not been proven to have the ability to against dengue and Zika infection. Thus, in this

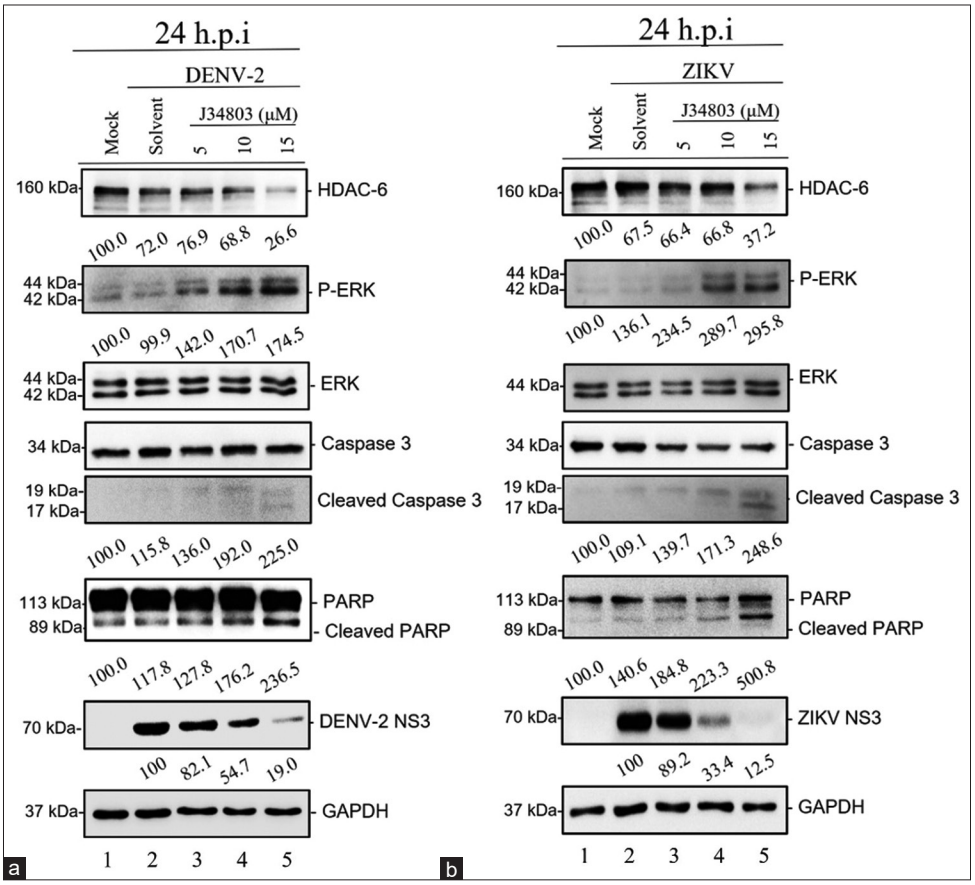

Figure 3: The compound J34803 suppressed DENV and ZIKV replication through the HDAC6 downstream signaling pathway. HEK-293 cells were infected with DENV-2 (a) or ZIKV (b) for 24 h (MOI = 1) and treated with 5, 10, or 15 μM of the compound J34803 to detect HDAC6, ERK, Caspase 3, and PARP signaling molecules by Western blotting. Relative ratios of HDAC6, p-ERK, cleaved Caspase 3 and cleaved PARP levels to HDAC6, ERK, Caspase 3, and PARP levels were adjusted to those of the mock control. Viral NS3 protein levels were also determined by Western blot analysis. The relative ratios of viral NS3 protein levels to GAPDH levels were adjusted to those of the solvent control. GAPDH was used as the loading control. DENV: Dengue virus, ZIKV: Zika virus, HDAC6: Histone deacetylase 6

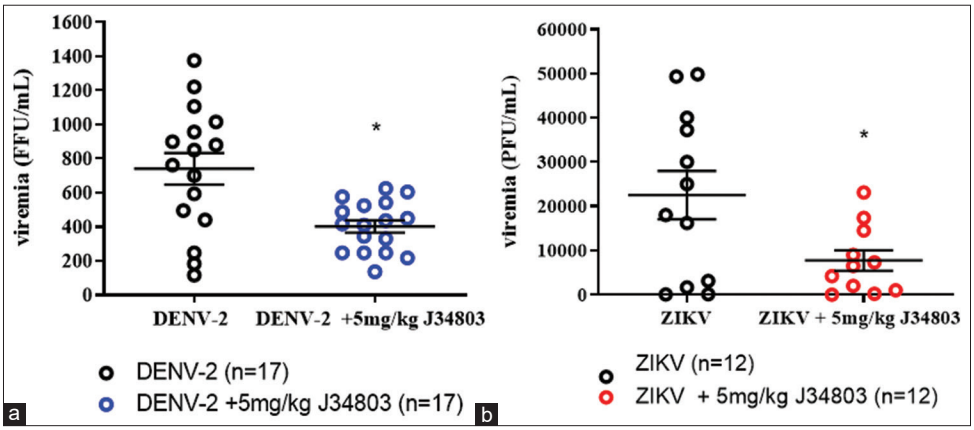

Figure 4: The compound J34803 exhibited protective efficacy *in vivo*. AG129 mice were infected with DENV-2 (a) or ZIKV (b) and treated without or with 5 mg/kg of the compound J34803 daily by intraperitoneal injection. Mouse sera were collected on day 3 after treatment. DENV-2 or ZIKV viremia levels were measured by focus-forming assays. According to two-tailed Student's *t*-test, $*P < 0.05$. DENV: Dengue virus, ZIKV: Zika virus

study, we used the compound J34803 [Figure 1a], a newly synthesized HDAC6 inhibitor, to evaluate its effect on DENV and ZIKV infection.

First, we measured the cell cytotoxicity of the compound J34803 [Figure 1b] and found the optimal concentrations for the subsequent experiments. We next investigated the effect of different concentrations of the compound J34803 and TBSA in HEK-293 cells with infection of DENV-2, DENV-4, or ZIKV (MOI = 1). The results showed that the compound J34803 exhibited superior anti-viral ability compared to TBSA [Figure 2].

As a previous study had shown that JEV infection may active PI3K/Akt signal through Bcl-2 mechanism to induce apoptosis of infected cells through Caspase-9 pathway during early stage of infection[38] and end up with apoptosis.[39] As shown in Figure 3, the compound J34803 efficiently inhibited DENV-2 and ZIKV infection in HEK-293 cells, and our results also showed that the compound J34803 not only inhibits HDAC6 expression but also promotes the activation of ERK and apoptosis proteins of mitochondria pathway, as well as the tendency of DENV and ZIKV proteins reduction.

In the animal test, the results showed that the treatment of the compound J34803 at 5 mg/kg daily efficiently reduced the viremia levels of DENV-2-and ZIKV-infected mice [Figure 4]. However, detailed studies on the optimal doses and dosing regimens of the compound J34803, either alone or in combination with other drugs, should be further examined to improve the compound's antiviral efficacy *in vivo*. Overall, our findings revealed that the compound J34803 exhibited better therapeutic efficacy *in vitro* and *in vivo* and could be applied in the treatment of emerging flaviviral infections that lack any identified therapeutic drugs.

CONCLUSION

In conclusion, we demonstrated that the compound J34803 had superior therapeutic efficacy in virus infection compared to TBSA and could be a potential potent therapeutic drug against emerging flaviviral infections.

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Data availability statement

The data that support the findings of this study are available from the corresponding author, KC Chiu, upon reasonable request.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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