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Short Communication

Phloretin inhibits Zika virus infection by interfering with cellular glucose utilisation

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ABSTRACT

Zika virus (ZIKV) is a re-emerging *Flavivirus* that has been linked to microcephaly and other neurological pathologies. In this study, phloretin, a glucose transporter inhibitor naturally derived from plants, was used to investigate the glucose dependence of ZIKV replication in host cells. The results showed that phloretin significantly decreased infectious titres of two ZIKV strains, namely MR766 (African genotype) and PRVABC59 (Puerto Rico genotype). The 50% effective concentration (EC_{50}) of phloretin against MR766 and PRVABC59 was 22.85 μM and 9.31 μM, respectively. Further analyses demonstrated that decreased viral production was due to host-targeted inhibition, including decreased apoptotic caspase-3 and -7 activities and reduced phosphorylation of Akt/mTOR pathways. In addition, upon disruption of cellular glucose availability within host cells using 2-deoxy-D-glucose, ZIKV propagation was inhibited. Collectively, we demonstrate phloretin inhibition of ZIKV propagation and provide evidence of glucose utilization pathways as being important for ZIKV propagation. The activity of phloretin and its role in inhibiting glucose uptake could provide a useful foundation for the development of ZIKV antivirals.

Zika virus (ZIKV) is a mosquito-borne virus first isolated in 1947 belonging to the *Flaviviridae* family. It is capable of causing severe congenital malformations such as microcephaly in infants and is linked to meningoencephalitis and Guillain–Barré syndrome in adults [1]. Epidemic ZIKV infection has escalated since the outbreak in Brazil in 2015, is widely spread throughout the world, and outbreaks in North America and Europe have been reported [2]. As such, the World Health Organization (WHO) declared ZIKV infection a Public Health Emergency of International Concern (PHEIC) in 2016. Despite the severity of the outbreak, there are currently no US Food and Drug Administration (FDA)-approved antiviral drugs or vaccines available to the public. Therefore, complementary and alternative strategies are needed to develop antiviral agents with activity against ZIKV.

It has recently been noted that increased glucose metabolism is associated with the pathogenesis and infectivity of Flaviviruses, such as dengue virus, which potentially contributes to the development of pancreatitis and diabetes mellitus along with dengue haemorrhagic fever [3,4]. A clinical observation from a ZIKV-infected patient with meningitis indicated an elevated ratio of cerebrospinal fluid-to-blood glucose [5]. This clinical manifestation could be associated with meningoencephalitis and the severity of other neurological disorders induced by ZIKV. As a result, impeding glucose metabolism is proposed to be a plausible approach to alleviate ZIKV infection [6]. In this study, the anti-ZIKV efficacy of the glucose transporter inhibitor phloretin, a naturally occurring antioxidant found in apple and pear trees, was explored. Both phloretin and its derivate phlorizin can bind competitively to glucose carriers, reducing glucose transportation into brain tissue [7]. Here we report the ability of phloretin to suppress ZIKV infection via interference with cellular homeostasis of glucose utilization in an *in vitro* model.

The antiviral activity of phloretin was first assessed by measuring its ability to inhibit ZIKV-induced cell death. Vero cells were infected with two ZIKV strains, namely MR766 (African strain) and PRVABC59 (Puerto Rico strain) at a multiplicity of

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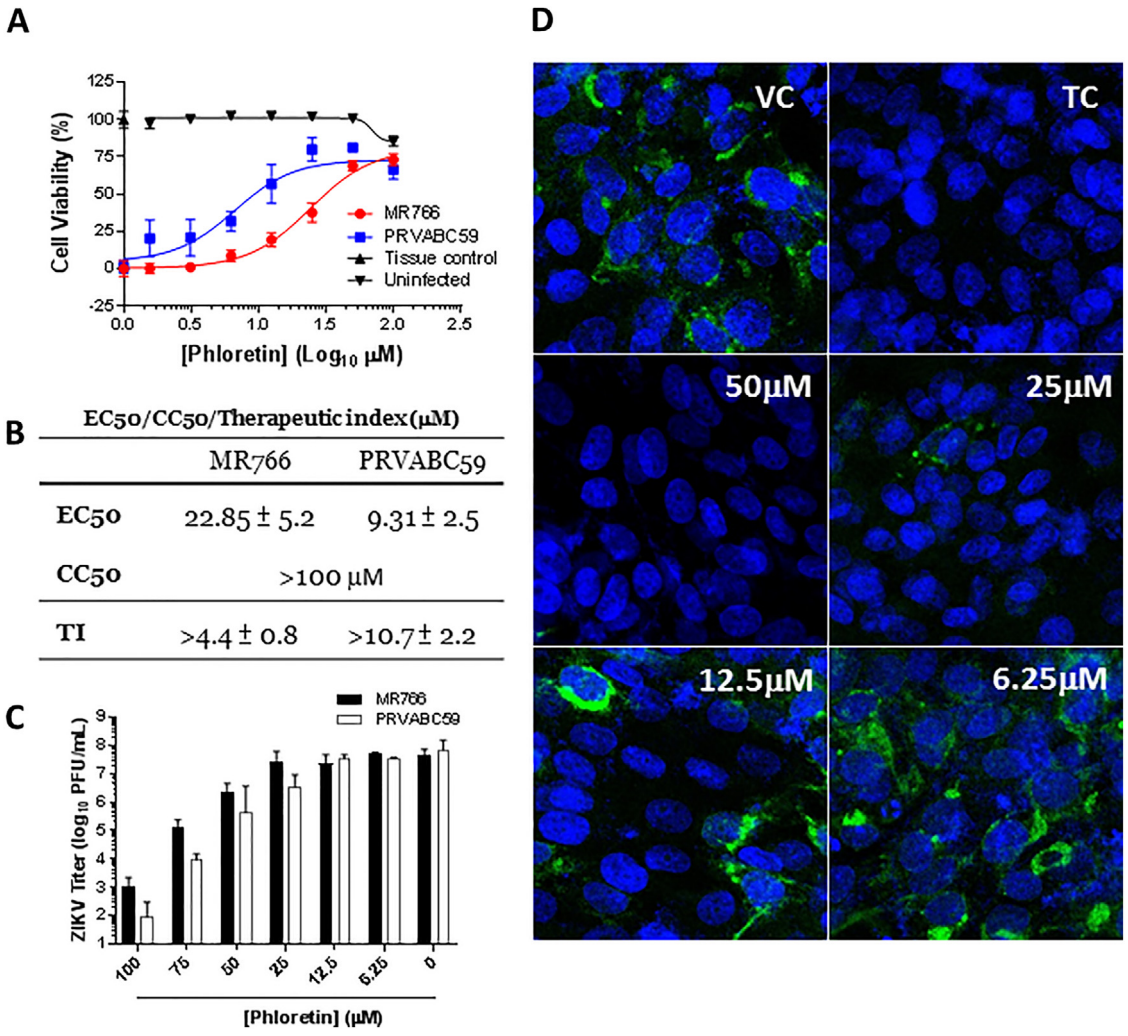

Fig. 1. Phloretin suppresses Zika virus (ZIKV)-induced cell death and ZIKV production. (A) Vero cells were pre-treated with phloretin for 1 h and were then infected with ZIKV MR766 or PRVABC59 at a multiplicity of infection (MOI) of 0.1. Following infection, the inoculum was removed and was replaced with medium containing fresh phloretin. Cell viability was determined using a CellTiter-Glo® Luminescent Cell Viability Assay at 72 h post-infection (hpi). Tissue control = uninfected, untreated cells. (B) Table of 50% effective concentration (EC_{50}), 50% cytotoxic concentration (CC_{50}) and therapeutic index ($TI = CC_{50}/EC_{50}$) values of phloretin against MR766 and PRVABC59. (C) Vero cells were treated and infected as in (A). Viral infectivity was measured by plaque assay at 48 hpi. Data are plotted as the mean ± standard error of the mean (SEM). (D) Vero cells were treated and infected as in (A). The distribution of intracellular ZIKV MR766 (green, E protein) at 48 hpi was assessed by confocal microscopy. Nuclei were stained with DAPI (blue). Magnification, 680×. TC, tissue control (= uninfected and untreated cells); VC, virus-only control.

infection (MOI) of 0.1. At 72 h post-infection (hpi), cellular viability was assessed using a CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison WI), a luminescence-based assay that quantifies the amount of ATP in metabolically active cells. Phloretin was applied at varying concentrations to cells for 1 h prior to ZIKV infection. Following infection, the inoculum was replaced with culture medium containing fresh phloretin until the experimental endpoint. This treatment regimen was used throughout all experiments unless otherwise noted. Phloretin restored up to 60% (MR766) and 80% (PRVABC59) of cell viability following ZIKV infection (Fig. 1A); the 50% effective concentration (EC_{50}) of phloretin against MR766 and PRVABC59 was 22.85 μM and 9.31 μM, respectively (Fig. 1B). Very little toxicity was noted in Vero cells treated with up to 100 μM of phloretin, therefore the 50% cytotoxic concentration (CC_{50}) was noted as being >100 μM (Fig. 1A,B), indicating that the inhibitory effect of phloretin was not due to cytotoxicity. To further explore the anti-ZIKV effectiveness of phloretin, cell viability of ZIKV-infected Vero cells at a MOI of 0.1 was measured after treating with phloretin for 96 h. Consistent with the data at 72 hpi, phloretin suppressed cellular death induced by ZIKV up to 96 h (Supplementary Fig. S1A).

To determine whether the increase in cell viability was related to a decrease in viral propagation, viral titres from the supernatant of ZIKV-infected cells collected at 48 hpi were assessed via plaque assay. Infectious titres of MR766 and PRVABC59 strains were significantly inhibited by phloretin in a dose-dependent manner (Fig. 1C). Furthermore, the anti-ZIKV activity induced by phloretin was confirmed through staining of intracellular ZIKV proteins with a 1:250 dilution of the anti-*Flavivirus* envelope glycoprotein antibody (Clone D1-4G2-4-15) and observing similar levels of viral inhibition (Fig. 1D) under confocal microscopy. To confirm that the anti-ZIKV activity of phloretin was not limited to Vero cells, cell viability and viral titres of ZIKV-infected U87MG astroglioma cells in the presence of phloretin were examined. Phloretin also reduced ZIKV-induced cell death and viral titres in U87MG cells with very slight cytotoxicity (Supplementary Fig. S2A, B, E). Phloretin treatment was not effective at reducing ZIKV-induced cell death in human umbilical vein endothelial cells (HUVECs) (Supplementary Fig. S2C). Whilst there was some reduction in viral titres at higher concentrations of phloretin, this can be attributed to the cell death observed in these samples (Supplementary Fig. S2C, D). These re-

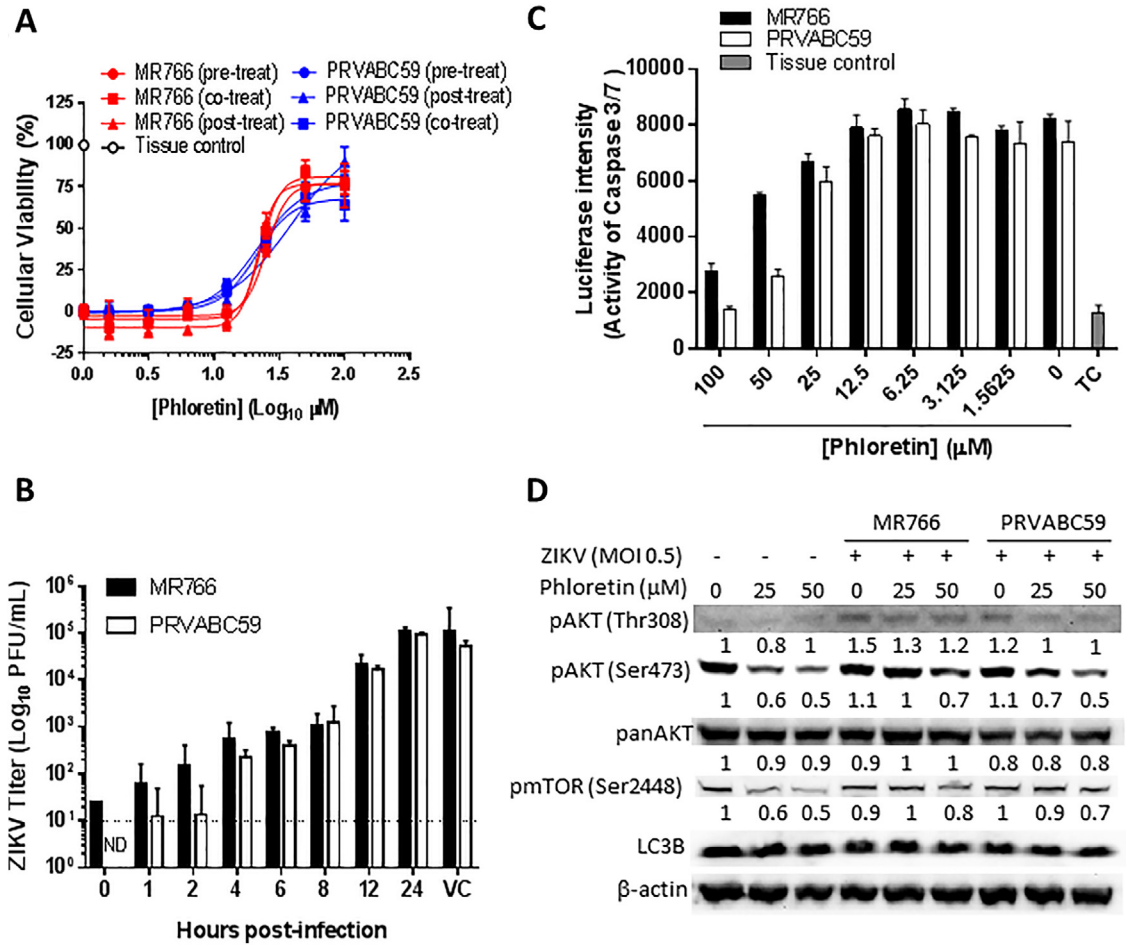

Fig. 2. Phloretin treatment inhibits multiple steps in Zika virus (ZIKV) replication and inhibits caspase-3 and -7 activities and Akt/mTOR signalling. (A) Phloretin was added either beforehand (pre-treat), simultaneously with infection (co-treat) or immediately after inoculum removal (post-treat). Vero cells were infected with either ZIKV MR766 or PRVABC59 at a multiplicity of infection (MOI) of 0.1. Cell viability was determined by CellTiter-Glo® Luminescent Cell Viability Assay at 72 h post-infection (hpi). (B) Phloretin (100 mM) was added to ZIKV-infected Vero cells (MOI of 0.5) at the indicated time point post-infection and was retained for the remainder of the experiment. Supernatants were collected at 24 hpi and viral titres were determined by plaque assay. Data are plotted as the mean ± standard error of the mean (SEM). (C) Vero cells were pre-treated with phloretin for 1 h and were then infected with ZIKV MR766 or PRVABC59 (MOI of 0.1). Following infection, the inoculum was removed and was replaced with medium containing fresh phloretin. Activities of caspase-3 and -7 were measured by Caspase-Glo® 3/7 Assay at 48 hpi. Data are plotted as the mean ± SEM. (D) Cells were treated as described in (C). Cells were infected with ZIKV (MOI of 0.5) and cell lysates collected at 24 hpi. Western blots were performed using antibodies against pAKT Thr308 (Cat# 9271; Cell Signaling Technology, Danvers, MA), pAKT Ser473 (Cat# 13038; Cell Signaling Technology), panAKT (Cat# 4691; Cell Signaling Technology), pmTOR Ser2448 (Cat# 5536; Cell Signaling Technology), LC3B (Cat# 3868; Cell Signaling Technology) and β-actin. Numbers below the blots are densitometry analysis of protein expression normalised to mock-infected untreated samples. LC3B, autophagic response protein; TC, tissue control (= uninfected and untreated cells); VC, virus-only control.

sults indicate that phloretin can inhibit ZIKV infection in Vero and U87MG cells.

Next, the potential mechanism of anti-ZIKV activity exerted by phloretin was explored through a time-of-addition analysis. Phloretin was added to Vero cells 1 h before ZIKV infection, simultaneously with ZIKV infection or 1 h post-infection. A CellTiter-Glo® Luminescent Cell Viability Assay was conducted at 72 hpi to assess cellular viability. There was a dose-dependent increase in the percentage of cellular viability upon treatment with phloretin that was independent of viral strain and the time of addition (Fig. 2A). ZIKV-infected cells were also treated with 100 μM phloretin at 0, 1, 2, 4, 6, 8, 12 and 24 hpi and viral titres were quantified by plaque assay. Delayed administration of phloretin to ZIKV-infected cells could still prevent ZIKV propagation but was not as efficient (Fig. 2B). These data suggest that phloretin inhibits ZIKV replication or propagation and that phloretin appears to have a therapeutic benefit against ZIKV even at later stages of infection after the virus has invaded host cells. To further delineate the anti-ZIKV activity of phloretin, viral RNA in the supernatants and cell lysates of MR766- and PRVABC59-infected cells under the same treatment conditions was quantified using quantitative reverse transcription PCR (RT-qPCR). RT-PCR was performed using an RNA UltraSense™ One-Step Quantitative RT-PCR Kit (Invitrogen) according to the manufacturer's instructions and using previously published primers and probe [8]. Following treatment with 100 μM phloretin, intracellular ZIKV RNA levels were reduced by ca. 2 log (Supplementary Fig. S1B), suggesting the ZIKV RNA replication may be affected. A 2.5–3 log reduction in extracellular RNA was observed following treatment with 100 μM phloretin (Supplementary Fig. S1C), which was consistent with the ca. 3 log reduction in viral titres (Fig. 1C). Collectively, these results suggest that phloretin may affect multiple stages of viral replication, including RNA production and a later stage of propagation such as assembly or egress.

As these results indicated that phloretin is a potent suppressor of ZIKV-induced cell death, we determined whether the inhibitory activity of phloretin was correlated with alterations in apoptotic pathways by measuring the activities of caspase-3 and -7 using a Caspase-Glo® 3/7 Assay (Promega). Caspase-3 and -7 activities in ZIKV-infected cells were increased, while phloretin treatment

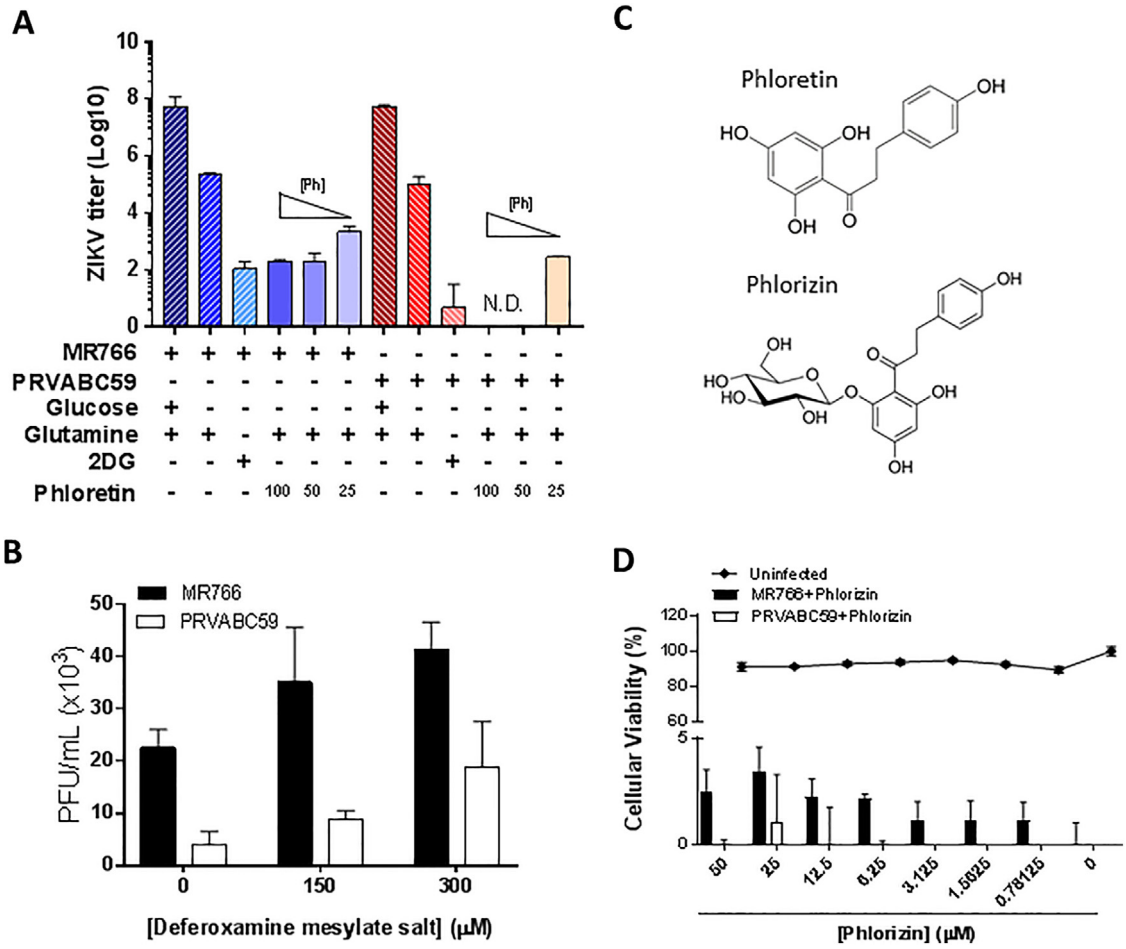

Fig. 3. Phloretin inhibits Zika virus (ZIKV) by interfering with cellular glucose utilization. (A) Vero cells were starved with serum-free medium for 16 h, followed by infection with ZIKV at a multiplicity of infection (MOI) of 0.5 in culture medium containing either glucose, 2 mM glutamine, 10 μM 2-deoxy-D-glucose (2DG) and/or phloretin. Titres were assessed at 24 h post-infection (hpi) by plaque assay. Data are plotted as the mean ± standard error of the mean (SEM). (B) Starved Vero cells were replete with glucose with or without deferoxamine mesylate salt for 6 h prior to infection. TC, tissue control (= uninfected and untreated cells); VC, virus-only control; ND, not detected. Data are plotted as the mean ± SEM. (C) Chemical structure of phloretin and phlorizin. (D) Vero cells were pre-treated with phlorizin for 1 h and were then infected with ZIKV MR766 or PRVABC59 (MOI of 0.1). Following infection, the inoculum was removed and was replaced with medium containing fresh phlorizin. Cell viability was determined by CellTiter-Glo® Luminescent Cell Viability Assay at 72 hpi.

substantially decreased the activities of caspase-3 and -7 in a dose-dependent manner by 48 hpi (Fig. 2C). Given that the Akt pathway regulates glycolysis and increases glucose metabolism [9,10] and that activation of the Akt pathway has recently been demonstrated to facilitate reprogramming of host cells by alphaviruses, enhancing their virulence [11], we reasoned that the Akt pathway could play an important role in the mechanism of action of phloretin to reduce ZIKV production. Thus, phosphorylation of Akt at Ser473 and Thr308 was examined according to previously established western blot protocols [12]. Phosphorylation of both residues was decreased in the presence of phloretin in a dose-dependent manner (Fig. 2D). In addition, phosphorylation of the downstream protein (mTOR) was subsequently decreased, confirming that the Akt/mTOR pathway might be one pathway altered in phloretin-treated cells to oppose ZIKV propagation (Fig. 2D). Of note, protein expression of LC3B, an autophagic response protein, remained unchanged following ZIKV infection and phloretin treatment, implying that the autophagic pathway may not be involved in this anti-ZIKV mechanism.

Given that phloretin is a traditional glucose transporter (GLUT1) inhibitor [13,14], we sought to investigate the role of glucose utilization in ZIKV-infected cells. Vero cells were serum-starved overnight to deplete the remaining cellular glucose prior to ZIKV infection, followed by the addition of 2% fetal bovine serum (FBS) culture medium containing glucose, 2 mM glutamine or 10 μM 2-deoxy-D-glucose (2DG), a hexokinase inhibitor, and/or different concentrations of phloretin. At 24 hpi, ZIKV titres decreased by ca. 2 log when cells were cultured in glucose-free, glutamine-supplemented medium, whereas an even greater reduction in viral titres was observed following addition of 2DG or phloretin in the culture medium (Fig. 3A). Notably, the decreasing viral titres were dose-dependently related to the dosage of phloretin, suggesting that glucose is required for ZIKV propagation, which could be disrupted by phloretin. To confirm the essential role of glucose utilisation for ZIKV, serum-starved cells were incubated with 150 μM and 300 μM deferoxamine (DFO) mesylate salt to mimic a cellular hypoxaemia condition that has been shown to increase the expression of GLUT1 [3]. As expected, viral titres of ZIKV were increased in a dose-dependent fashion in conjunction with DFO treatment, suggesting that the increased ZIKV viral titres could be associated with GLUT1 expression (Fig. 3B).

Next, the impact of phlorizin (phloretin 2′-O-glucose; Fig. 3C), a glycosidic form of phloretin, on ZIKV replication was assessed. Phlorizin is an inhibitor of Na^+ and glucose co-transporter SGLT1 and SGLT2 and possesses similar physiological effects as phloretin [15,16]. Therefore, we next tested whether phlorizin exhibited sim-

ilar anti-ZIKV activity by evaluating the viability of ZIKV-infected cells treated with phlorizin. Interestingly, phlorizin showed limited cellular protection against both strains of ZIKV with slight cytotoxicity upon phlorizin treatment (Fig. 3D). Although a phloretin derivative, there was significantly decreased anti-ZIKV activity in infected cells treated with phlorizin. There is potential that the additional glucoside group, unique to phlorizin, which is conjugated to the conserved backbone between the two compounds, alters its substrate specificity and subsequent anti-ZIKV activity. This is already partially observed as phlorizin has a preference for inhibition of Na^{2+}-dependent glucose transporters (e.g. SGLT1), whereas phloretin inhibits Na^{2+}-independent glucose transporters (e.g. GLUT1). This is an area of interest for future research.

Collectively, these data demonstrate that the anti-ZIKV activity of phloretin is not strain-specific as phloretin suppressed both MR766 and PRVABC59 replication. In addition, the anti-ZIKV activity of phloretin may be due to inhibition of viral replication or modulation of antiapoptotic activity as exhibited in the down-regulation of caspase-3 and -7 activities. We also confirmed that glucose metabolism is required for ZIKV propagation in host cells, which endows phloretin with anti-ZIKV activity as summarised in Supplementary Fig. S3. Overall, these results suggest that phloretin warrants further study for evaluation of anti-ZIKV efficacy in addition to further elucidation of the role of glucose pathways in the modulation of ZIKV activities.

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Competing interests

None declared.

Ethical approval

Not required.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2019.03.017.

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