LETTER

Antiviral activity of quercetin-3-β-O-D-glucoside against Zika virus infection

Dear Editor,

The 2015–2016 outbreak of Zika virus (ZIKV) fever, first reported in Brazil during early 2015 (Zanluca et al., 2015), has infected millions of people and is a global public health concern. ZIKV infections are associated with fetal microcephaly, as well as neurological complications in humans. The virus can be shed in the semen and vaginal secretions of humans, leading to sexual transmission, and unexpectedly ZIKV infections cause severe damage to the male reproductive organs in male mice (Govero et al., 2016; Ma et al., 2016). Medical countermeasures to ZIKV are urgently needed. Vaccines and antivirals, such as monoclonal antibodies (mAbs), are currently in pre-clinical development and not yet approved for human use. There have been concerns that vaccines and mAb-based products against ZIKV may inadvertently enhance the severity of Dengue virus (DENV) infections (Kawiecki and Christofferson, 2016; Richner et al., 2017), a potentially dangerous undesired side effect. Therefore, the development of small molecule drugs is a priority for controlling and preventing the spread of ZIKV.

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is one of the most ubiquitous flavonoids, found in abundance in many Chinese herbs, vegetables, and fruits, as well as in red wine (Harwood et al., 2007). Quercetin has been shown to exert anti-proliferative, anti-oxidative, anti-bacterial, anti-cancer, and anti-viral effects; it was previously shown to have a beneficial effect on patients with severe complications from pandemic influenza A (H1N1) virus infection (Uchide and Toyoda, 2011). Quercetin-3-β-O-D-glucoside (Q3G, molecular weight = 464.38) is a natural derivative of quercetin containing a glucoside molecule (Supplementary Figure S1A), which shows enhanced solubility and bioavailability when ingested (Hollman and Katan, 1997). Previous studies on quercetin and its derivatives focused on their functional properties with regard to potential therapeutic activity against cancer or inflammatory and anti-metabolic disease (D'Andrea, 2015). Recent studies have also demonstrated the antiviral activity of these compounds against a number of pathogens, including Influenza virus, Chikungunya virus, Epstein-Barr virus, Hepatitis B virus, Hepatitis C virus, Mayaro virus, and Ebola virus (Cheng, et al., 2015; Qiu et al., 2016).

In this study, we investigated the anti-ZIKV effects of Q3G on Vero cells *in vitro*. In addition, the *in vivo* effects of Q3G were also evaluated in immunocompromised mice lacking the receptor for type I interferon (Jackson laboratories, 32045-JAX, Bar Harbour, ME), hereafter referred to as *Ifnar1*$^{-/-}$. Q3G was obtained commercially (Sigma-Aldrich, 17793, Oakville, Canada). Two different ZIKV strains from the Asian lineage, which were isolated from laboratory-confirmed patients, were used for this study; PLCal_ZV (GenBank KF993678.1) was used for the *in vitro* experiments and PRVABC59 (GenBank KU501215) was used for the *in vivo* experiments. Stock viruses were grown in Vero cells (ATCC, CCL-81, Manassas, VA), and stored at –80 °C. All experiments were conducted in the Biosafety Level 2 (BSL-2) Laboratory at the National Microbiology Laboratory, Winnipeg, Canada, and animal experiments were carried out in strict accordance with the recommendations of Canadian Council on Animal Care (CCAC), with approval from the Animal Care Committee (ACC).

First, we evaluated the *in vitro* antiviral activity of Q3G. Our previous study had shown that treatment of Vero E6 cells with less than 100 μmol/L of Q3G does not result in cytotoxicity (Qiu et al., 2016). In the current study, Vero cells were pre-treated with 2-fold serially diluted Q3G (ranging from 0.67 μmol/L to 10.76 μmol/L) or DMSO (control) for 1 h at 37 °C, and infected with PLCal_ZV at a multiplicity of infection (MOI) of 0.05. After 1 h, the inoculum was removed, and MEM supplemented with 1% penicillin-streptomycin, 1% fetal bovine serum, and Q3G or DMSO was added. The viral RNA was extracted from the supernatant using the QIAamp viral RNA minikit (Qiagen, 52906, Toronto, Canada) at 2 or 4 days post-infection (dpi), and quantified by quantitative reverse transcription PCR (RT-qPCR) using the Light-Cycler 480 RNA Master Hydrolysis Probes kit (Roche, 04991885001, Mississauga, Canada) and a primer-probe set (Probe860), as reported previously (Lanciotti et al., 2008). The reaction conditions were as follows: 63 °C for 3 min; 95 °C for 30 s; and 45 cycles of 95 °C for 15 s and 60 °C for 30 s. The reactions were performed on an

ABI Step OnePlus thermocycler. The results for Q3G were normalized to the control (set at 0% inhibition). The 50% effective concentration (EC_{50}) and 90% effective concentration (EC_{90}) values were calculated using a four-parameter logistic regression in Prism 5 (GraphPad, La Jolla, CA). The EC_{50} and EC_{90} of Q3G against PLCal_ZV were approximately 1.2–1.3 µmol/L and 1.5 µmol/L, respectively, when assessed at both 2 and 4 dpi (Figure 1A). The Q3G-mediated inhibition of PLCal_ZV (MOI = 0.05) in Vero cells was also confirmed visually under a microscope, and complete inhibition of cytopathic effects (CPE) was observed at a concentration of 6.9 µmol/L at 4 dpi (Figure S1B).

We then used the same *in vitro* infection conditions to quantify the expression of ZIKV nonstructural protein 1 (NS1) at 4 dpi, in order to estimate virus replication at the protein level. NS1 is known to be a major host-interaction molecule involved in virus replication, pathogenesis, and immune evasion (Rastogi et al., 2016). Using an anti-ZIKV NS1 ELISA kit (BioFront Technologies, ZIKV-NS-1-EK, Tallahassee, FL), we showed that Q3G could inhibit the replication of PLCal_ZV in a dose-dependent manner, as evidenced by the decrease in NS1 in both the cell supernatant and lysate, with statistically significant

(as determined by Student's *t*-test) reductions observed at concentrations above 0.6 µmol/L (Figure 1B, 1C).

Next, we tested the protective efficacy of Q3G in immuno-compromised mice (*Ifnar1*^{-/-}) that were previously shown to be susceptible to ZIKV infection (Lazear et al., 2016). Our previous study had shown that the administration of Q3G at doses of 100 mg/kg or lower to mice do not result in substantial weight loss or death (Qiu et al., 2016). In the current study, 1×10^6 plaque forming units (PFU)/animal of the PRVABC59 isolate of ZIKV was intraperitoneally injected into 6- to 8-week old male or female *Ifnar1*^{-/-} mice (n = 6 per group) (day 0) ; the mice were then observed daily for survival and weight loss. We administered Q3G via IP at a dose of 50 mg/kg beginning at 1 day after challenge (day 1) and continuing for 7 days (day 8). Control animals were administered the same volume of PBS under the same regimens (Figure 1D). The control animals uniformly succumbed to ZIKV infection at 7 dpi with an average of ~20% weight loss at the time of death (Figure 1E, 1F). Post-exposure administration of Q3G was partially effective, with 50% of the animals surviving the infection and an average weight loss of ~20% (Figure 1E, 1F). Administration of Q3G via IP at a dose of 50 mg/kg beginning at 1 day
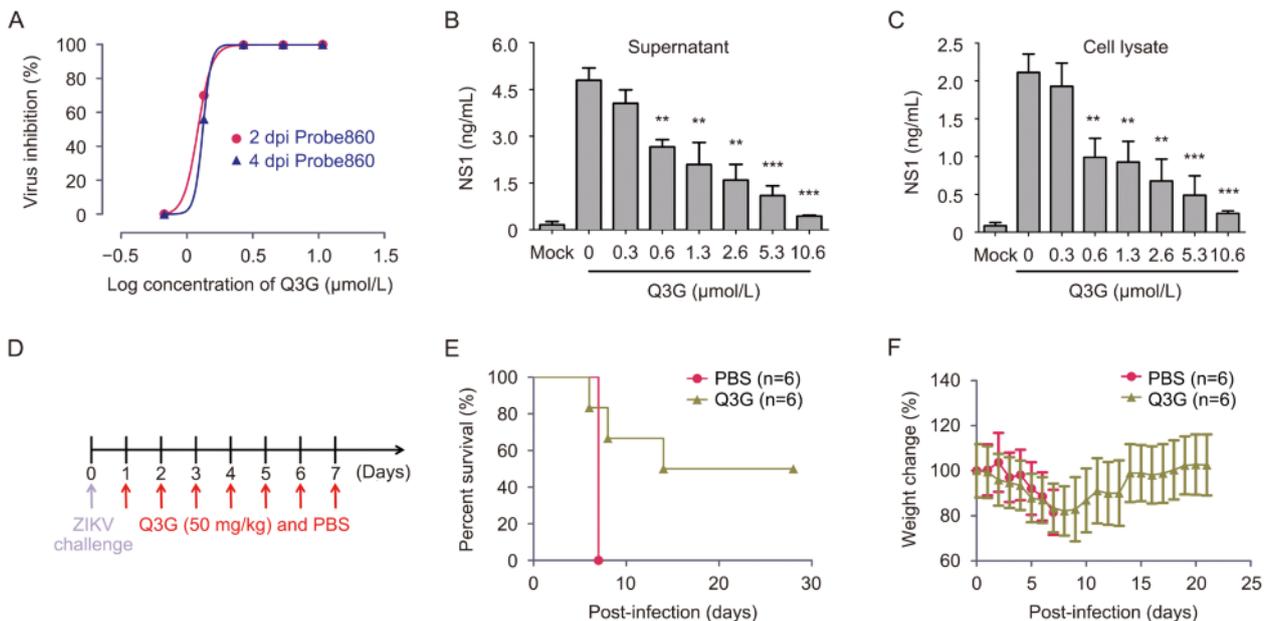

Figure 1. Antiviral activity of Q3G against ZIKV in cell culture and treatment of ZIKV-infected *Ifnar1*^{-/-} mice with Q3G. Viral inhibition curves of various concentrations of Q3G against ZIKV PLCal_ZV assessed at (A) 2 days post-infection (pink circles) or 4 days post-infection (blue triangles). Quantification of ZIKV NS1 expression by ELISA from the (B) supernatant and (C) cell lysate of Vero cells infected with PLCal_ZV and treated with various concentrations of Q3G. "Mock" indicates mock infection. All experiments were performed in triplicate, and error bars represent mean ± standard deviation. ** $P < 0.01$; *** $P < 0.001$. (D) Post-exposure treatment schedule. (E) Survival and (F) weight change over time. Mice (n = 6/group) were treated with either PBS (pink circles) or Q3G (green triangles) and challenged intraperitoneally with 1×10^6 PFU of the PRVABC59 isolate. Error bars indicate standard error of the mean.

before challenge (day -1) and continuing for 7 days (day 5) yielded neither survivors nor a substantial delay in the time to death (data not shown).

During the 2014–2016 outbreak of Ebola virus disease, clinically licensed or near-approved drugs for other diseases were repurposed to treat Ebola out of desperation, despite the lack of efficacy data in some cases (Wong and Kobinger, 2015), as the compounds were already shown to be safe for human use. The proactive generation of pre-clinical efficacy data on licensed or near-approved drugs against other pathogens in cell cultures and animal models is therefore important, as it provides medical professionals a scientific basis to make informed decisions for treatment options in case of emergencies. Q3G is a natural derivative of quercetin and is already widely used in various foods and drinks. Our results clearly demonstrated that Q3G exerts antiviral activity against ZIKV in both tissue culture and knockout mice, and that post-exposure *in vivo* treatment with Q3G could have a beneficial effect. In the future, Q3G should be tested in human cell lines (such as Huh-7, HeLa, or K048, a fetal brain neural stem cell line) to provide further data supporting its potential efficacy in humans; in addition, live viral loads or viremia should be tested in treated animals to supplement the survival results observed in this study. Although the treatment regimens will need to be further optimized (i.e., dosage, frequency of treatment, and administration routes), our results support the results of Q3G efficacy studies in nonhuman primates against ZIKV infection. Further studies will also be needed to investigate the mechanism of Q3G antiviral action, in order to obtain valuable insights into the design of novel targets for antiviral therapeutics in the future.

FOOTNOTES

Supplementary Figure S1 is available on the websites of *Virologica Sinica*: www.virosin.org; link.springer.com/journal/12250.

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