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Mefenamic acid in combination with ribavirin shows significant effects in reducing chikungunya virus infection *in vitro* and *in vivo*

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ABSTRACT

Chikungunya virus (CHIKV) infection is a persistent problem worldwide due to efficient adaptation of the viral vectors, *Aedes aegypti and Aedes albopictus* mosquitoes. Therefore, the absence of effective anti-CHIKV drugs to combat chikungunya outbreaks often leads to a significant impact on public health care. In this study, we investigated the antiviral activity of drugs that are used to alleviate infection symptoms, namely, the non-steroidal anti-inflammatory drugs (NSAIDs), on the premise that active compounds with potential antiviral and anti-inflammatory activities could be directly subjected for human use to treat CHIKV infections. Amongst the various NSAID compounds, Mefenamic acid (MEFE) and Meclofenamic acid (MECLO) showed considerable antiviral activity against viral replication individually or in combination with the common antiviral drug, Ribavirin (RIBA). The 50% effective concentration (EC_{50}) was estimated to be 13 μM for MEFE, 18 μM for MECLO and 10 μM for RIBA, while MEFE + RIBA (1:1) exhibited an EC_{50} of 3 μM, and MECLO + RIBA (1:1) was 5 μM. Because MEFE is commercially available and its synthesis is easier compared with MECLO, MEFE was selected for further *in vivo* antiviral activity analysis. Treatment with MEFE + RIBA resulted in a significant reduction of hypertrophic effects by CHIKV on the mouse liver and spleen. Viral titre quantification in the blood of CHIKV-infected mice through the plaque formation assay revealed that treatment with MEFE + RIBA exhibited a 6.5-fold reduction compared with untreated controls. In conclusion, our study demonstrated that MEFE in combination with RIBA exhibited significant anti-CHIKV activity by impairing viral replication *in vitro* and *in vivo*. Indeed, this finding may lead to an even broader application of these combinatorial treatments against other viral infections.

1. Introduction

Chikungunya virus (CHIKV) is a mosquito-transmitted alphavirus that belongs to the *Togaviridae* family (Mason and Haddow, 1957). CHIKV is serologically listed in the Semliki Forest complex of alphaviruses (Powers et al., 2001). CHIKV has spread throughout various regions in Africa and Asia. The first massive outbreak was reported in La Réunion Island and the Indian Ocean in 2005 followed by an outbreak in India, which drew the attention of the Western world (Pialoux et al., 2007). In the La Réunion Island case, CHIKV transmission was mostly caused by a secondary mosquito vector of the virus, *Aedes albopictus* (Reiter et al., 2006; Vazeille et al., 2007). Studies revealed that the efficient adaptation of *A. albopictus* was due to a mutation in the E1 glycoprotein (A226V) of the *A. albopictus* midgut cells, which increased the infectivity of the virus (Vazeille et al., 2007; Tsetsarkin et al., 2007). Meanwhile, in India, it was estimated that the virus has attacked 1.4 million residents, whilst mortality has been observed among infants and patients with multiple co-morbidities (Gerardin et al., 2008). A small outbreak was reported in Italy, which started from a CHIKV-

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viraemic traveller returning from a visit to India (Rezza et al., 2007). The outbreak apparently demonstrated that certain vector-borne viruses are competent to migrate into non-epidemic regions if they are exposed to suitable ecologic conditions (Chretien and Linthicum, 2007). In 2015, 7942 CHIKV infection cases have been confirmed in North America (WHO, 2015).

The CHIKV genome is a positive sense RNA that is approximately 11.8 Kb. The surface of enveloped virion contains 80 membrane-bound trimeric spikes, each of which is composed of triplet heterodimers of the glycoproteins, envelope 1 and 2 (E1 and E2) (Mukhopadhyay et al., 2006). CHIKV has been reported to infect and replicate actively in various cell types, including epithelial cells, endothelial cells and monocytes that originate from macrophages (Sourisseau et al., 2007; Ozden et al., 2007). Infection is shown to occur through pH dependent endocytosis using a receptor in clathrin-coated vesicles (Sourisseau et al., 2007; Rashad et al., 2014). The E2 glycoprotein is normally responsible for receptor binding, while E1 is involved in cell fusion (Schwartz and Albert, 2010). Recent studies demonstrated that anti-CHIKV IgG could be detected at the early phase of infection in patients' plasma. As such, the conformational changes in the E2 epitope represents a drug target in order to neutralize the alphavirus infection (Strauss and Strauss, 1994; Nowak et al., 1995). Thus, researchers have proposed the use of naturally acquired IgG (specifically IgG3 subclass) to target single-linear epitopes of the E2 glycoprotein (E2EP3), which is present on the viral envelope (Kam et al., 2012).

The absence of an anti-CHIKV vaccine or drugs caused the treatment strategies against CHIKV infection to be targeted only toward alleviating the symptoms that are associated with the infection. Non-steroidal anti-inflammatory drugs (NSAIDs) are the best drug candidates to alleviate viral infection symptoms, such as musculoskeletal disorders, inflammation and pain. We hypothesized that NSAIDs could be drugs with dual effects and not only cause symptom alleviation but also suppress CHIKV replication, especially in combination with common antiviral drugs, such as RIBA and ACIC. In this study, we confirmed that Mefenamic acid, a primary compound in the NSAID group, has potential antiviral activity *in vitro* and *in vivo*, and this activity is better achieved when delivered in combination with the common antiviral drug, RIBA.

2. Methods

2.1. Virus, cells and compounds

CHIKV was isolated from the serum sample of a patient who received hospital care and recovered. All compounds, including Mefenamic acid (MEFE), Meclofenamic acid (MECLO), Flufenamic acid (FLUFE), Tolfenamic acid (TOLF), RIBA and ACIC were purchased from Sigma, USA. The compounds were dissolved in dimethyl sulfoxide (DMSO) and the final concentration of DMSO in the tissue culture media was kept below 1% of the total volume in all of the subsequent experiments.

2.2. Maximum non-toxic dose (MNTD) test

Vero cells were seeded into 96-well plates at a density of 1×10^4 cells/well and treated with increasing concentrations of test compounds (12.5, 25, 50, 100, 200 and 400 μM) in prepared in Dulbecco's Modified Eagle Medium (DMEM) that was supplemented with 2% foetal bovine serum (FBS). After 72 h, the cell culture was analysed using a non-radioactive cell proliferation assay (Promega, USA) according to the manufacturer's instructions. The cell viability percentage was calculated as follows: (Absorbance of treated cells/Absorbance of untreated cells) × 100.

2.3. Evaluation of antiviral activities

To evaluate the antiviral activity of the test compounds, Vero cells were seeded into 24-well microplates (1.5×10^5 cells/well) and incubated for 24 h at 37 °C and 5% CO_2. The cells were infected with CHIKV at an MOI of 1 and later separately treated with test compounds (25 μM each) or a mixture (1:1) of RIBA + MEFE (12.5 μM each) or RIBA + MECLO (12.5 μM each) for 72 h. Next, a plaque formation assay was used to calculate the viral titre in the culture medium.

2.4. ELISA-like cell-based assay

To determine the 50% effective concentration (EC_{50}) of the test compounds, Vero cells were seeded in 96-well tissue culture plates (1×10^4 cells/well) and infected with CHIKV at an MOI of 1. After removing the media that was used for infection and washing the cells with PBS, test compounds were added at concentrations of 0 (positive control), 5, 10, 15, 20 and 25 μM, while combinations of MECLO + RIBA or MEFE + RIBA were administered at a ratio of 1:1 with a final concentration that was similar to the individual compound treatments. DMSO (1%) was used as a vehicle control. The infected cells were incubated in the presence of the test compounds at 37 °C and 5% CO_2 for 72 h.

Next, the cells were washed three times with PBS and fixed with ice-cold methanol for 15 min at −20 °C and incubated with blocking buffer. Later, a CHIKV antibody (Abcam, UK) was added, and the cells were incubated overnight at 4 °C. The cells were washed with PBS and incubated for 30 min with anti-mouse IgG that was conjugated with alkaline phosphatase. After adding the alkaline phosphatase substrate, the absorbance between 490 and 650 nm was measured using an ELISA reader. The EC_{50} values were calculated using non-linear regression fitting (GraphPad Prism, version 5.01).

2.5. Viral inactivation assay

MEFE, MECLO, RIBA (25 μM each) or DMSO (1%), as a vehicle control, were separately mixed with CHIKV (10^4 PFU) and incubated for 1 h at 37 °C in cell culture medium. The mixture was then diluted 50-fold with DMEM containing 2% FBS. Next, 100 PFU/well was added separately on each well of the Vero cells that were grown in 24-well plates. For comparison, test compound − virus mixtures were added to the cells without pre-incubation. Before discarding the compound-virus mixture, the cells were incubated for 1 h at 37 °C for virus adsorption. Then, the cells were washed 3 times with PBS and overlaid with DMEM containing 1.1% methylcellulose. Five days post-infection, virus plaques were revealed as described below and counted.

2.6. Viral attachment assay

To determine the inhibitory effect of the test compounds on virus attachment to the Vero cells, the cells were seeded in 96-well plates (1×10^4 cells/well), incubated at 4 °C for 1 h and subsequently inoculated with CHIKV (MOI of 1) in the presence of MEFE, MECLO, RIBA (0 [positive control], 5, 10, 15 and 25 μM each) or DMSO (1%) control for another 3 h at 4 °C. Then, the cells were washed three times with PBS and fixed with ice-cold methanol to proceed with the ELISA-like cell-based assay, as described above.

2.7. CHIKV replicon cell-based assay

Huh-7 cells that expressed *Renilla* luciferase (*Rluc*) by a CHIKV replicon were prepared as described by (Utt et al., 2015). The cells