



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



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ARTICLE INFO

Article history:

Received 27 April 2015

Received in revised form

2 December 2015

Accepted 14 January 2016

Available online 18 January 2016

Keywords:

Chikungunya virus

Non-steroidal anti-inflammatory drugs

Mefenamic acid

Meclofenamic acid

Ribavirin

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 (EC₅₀) was estimated to be 13 μM for MEFE, 18 μM for MECLO and 10 μM for RIBA, while MEFE + RIBA (1:1) exhibited an EC₅₀ 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.

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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 μ M) 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) \times 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% CO₂. The cells were infected with CHIKV at an MOI of 1 and later separately treated with test compounds (25 μ M each) or a mixture (1:1) of RIBA + MEFE (12.5 μ M each) or RIBA + MECLO (12.5 μ M 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 (EC₅₀) 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 μ M, 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% CO₂ 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 EC₅₀ values were calculated using non-linear regression fitting (GraphPad Prism, version 5.01).

2.5. Viral inactivation assay

MEFE, MECLO, RIBA (25 μ M 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 μ M 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

were seeded in a 96-well plate (1.4×10^4 /well) and treated with the test compounds (5, 15 and 25 μM) or DMSO (1%) as a control. After 48 h of incubation at standard conditions (37 °C and 5% CO_2), the *Rluc* luminescence signal was measured with a *Renilla* luciferase assay kit (Promega, WI, USA) and read on a GloMAX 20/20 Luminometer (Promega, WI, USA), according to the manufacturer's protocols. The data are represented as means and standard deviation of the mean (SD) from triplicate assays from three independent experiments.

2.8. Virus quantification with a plaque formation assay

CHIKV- infected cell supernatants were diluted 10-fold with DMEM containing 2% FBS and added to confluent Vero cells that were grown in 24-well plates (1.5×10^5 cells). The cells were incubated for 1 h at 37 °C and were overlaid with DMEM containing 1.1% methylcellulose. The viral plaques were revealed by staining with crystal violet dye after a 5-day incubation. The viral titres were calculated according to the following formula: Titre (PFU/ml) = plaque number/volume of the diluted virus added to the well \times the dilution factor of the virus that was used to infect the cells in the wells in which the plaques were enumerated.

2.9. In vivo antiviral and toxicity testing

Adult mice (ICR strain) that were used as animal models for this study were in accordance with the University of Malaya guidelines on the Care and Use of Laboratory Animals, and the study protocols applied in this study had been approved by the Animal Ethics Committee of the University of Malaya. An acute toxicity test was performed by intraperitoneally administering the animals with low (5 mg/kg) and high doses (50 mg/kg) of the test compounds individually, or in the stated combinations. The animals were observed for 24 h for signs of toxicity, and after 14 days post-treatment, the animals were sacrificed for histological examinations. Then, new animals (five groups, $n = 8$ each group) were used to study the efficacy of the compounds in the alleviation of CHIKV infection. The animal groups were intraperitoneally inoculated with 1×10^6 PFU of purified CHIKV, as previously described (Parashar et al., 2013). At 6 h post-infection, a single dose of the compounds was administered intraperitoneally to the groups that included the RIBA-treated group (infected-animals treated with 15 mg/kg of RIBA), the MEFE-treated group (infected-animals treated with 15 mg/kg of MEFE) and the MEFE + RIBA-treated group (infected-animals treated with 15 mg/kg of RIBA and 15 mg/kg of MEFE). The non-infected animals and untreated animals were administered with PBS as negative and positive mock-administered control groups, respectively. The mice were observed each day for 7 days post-infection and sacrificed for virus quantification and pathological analysis.

2.10. Statistical analysis

All of the assays were performed in triplicate, and statistical analyses were performed using GraphPad Prism, version 5.01 (GraphPad Software, San Diego, CA). P values that were <0.05 were considered to be statistically significant values.

3. Results

3.1. MEFE and MECLO inhibit CHIKV replication

The MTT assay results indicated that treatment with 25 μM of each drug for 72 h showed minimal effects on cell viability. Furthermore, amongst the test compounds, ACIC and RIBA showed minor effects on cell viability, even at high concentrations (Fig. S1).

In contrast, all of the test compounds (MEFE, MECLO, FLUFE, TOLE, ACIC, RIBA) showed activity against CHIKV infection. MECLO and MEFE showed the highest antiviral activity amongst the NSAID compounds (Fig. 1A and B) while RIBA showed higher antiviral activity compared with ACIC. Therefore, we sought to test the activity of MEFE + RIBA and MECLO + RIBA combinations against CHIKV infection. The combined treatments yielded higher antiviral activity compared with the individual treatments. MEFE + RIBA significantly (One way ANOVA, $P < 0.01$) reduced the viral titres (0.30×10^7 PFU/ml) compared with RIBA (0.94×10^7 PFU/ml) or MEFE (0.99×10^7 PFU/ml) alone, which was similar to the antiviral potential of MECLO + RIBA (0.31×10^7 PFU/ml) compared with MECLO alone (1.1×10^7 PFU/ml), as presented in Fig. 1A.

3.2. Combinations of MEFE + RIBA and MECLO + RIBA lead to considerable CHIKV reduction in Vero cells

An ELISA-like cell-based assay was used to evaluate the efficacy of MEFE and MECLO, individually or in combination with RIBA, in reducing the viral structural protein levels in infected cells. Our results showed that the test compounds inhibited CHIKV replication in Vero cells in a dose-dependent manner (Fig. 1C). 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) had an EC_{50} of 3 μM and MECLO + RIBA (1:1) had an EC_{50} of 5 μM . The results again demonstrated that the test compound combinations led to a considerable improvement in the reduction of CHIKV structural protein levels in Vero cells.

3.3. MEFE and MECLO inhibit CHIKV entry by inactivating viral particles and preventing virus binding and internalization into Vero cells

We incubated CHIKV with the test compound and then inoculated the cells to examine whether the compounds could inactivate CHIK virions and inhibit subsequent events of viral entry. Using a plaque reduction assay, the viral inhibition percentage significantly (Two-Way ANOVA, $p < 0.001$) depended upon the incubation of CHIKV with the test compound. After a 1 h incubation of the CHIKV-test compound mixture, MEFE and MECLO showed 85% and 78% reduction in numbers of formed plaques, respectively, compared with the mixture without incubation controls, which showed only 22% and 17% inhibition, respectively. Both compounds showed higher inhibition percentages compared with RIBA, while no significant difference was observed between MEFE and MECLO (Fig. 2A). This result suggested that MEFE and MECLO could bind to virus particles and neutralize the viral infectivity.

Next, we sought to test the ability of MEFE and MECLO to prevent viral attachment and penetration. An attachment assay was carried out at 4 °C that allowed for virus binding but prevented entry, which occurs most efficiently at 37 °C. Using an ELISA-like cell-based assay to detect the bound virus on the Vero cell monolayers, both compounds inhibited CHIKV attachment to the cell surface in a dose-dependent manner. Additionally, both compounds showed approximately 50% inhibition at a concentration of 15 μM , which was similar to the EC_{50} values (Fig. 2B). These results suggested that MEFE and MECLO might interact with the viral E2 envelope glycoprotein or virus receptors on the target cells. To test the inhibitory effect of MEFE and MECLO on virus penetration, a plaque reduction assay was used. For this experiment, CHIKV particles were incubated with Vero cells at 4 °C for optimal virus-cell binding without active penetration. Next, the virus was induced to penetrate the Vero cells by shifting the temperature to 37 °C in the presence of the compounds or 1% DMSO, as a negative (vehicle) control. It was determined that MEFE or MECLO treatment resulted

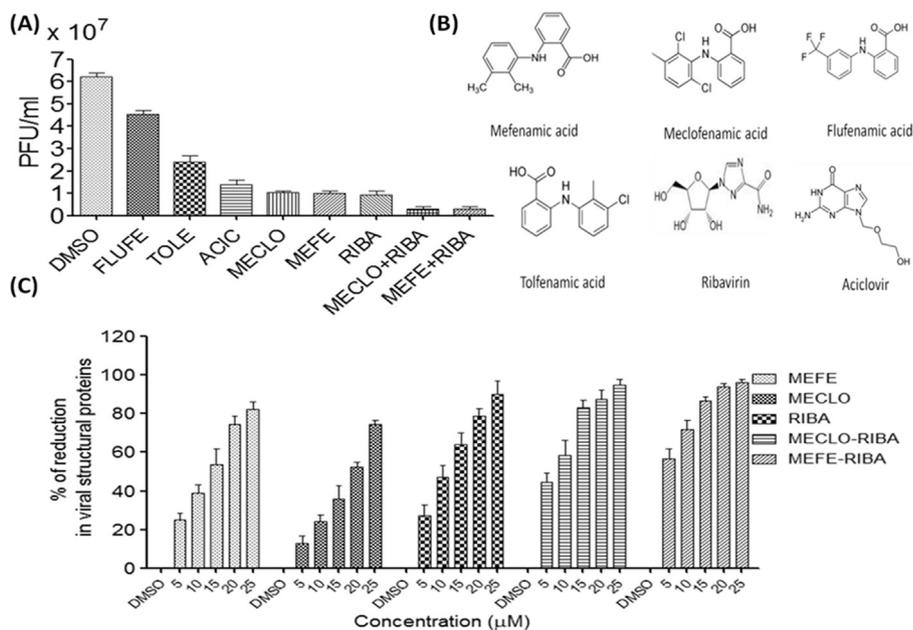


Fig. 1. The inhibitory effect of antimicrobial agents against CHIKV replication in Vero cells. (A) Vero cells were grown in 24-well microplates and incubated for 24 h at 37 °C and 5% CO₂. The cells were infected with CHIKV at an MOI of 1 and then separately treated with the test compounds (25 μM each) or mixture (1:1) of RIBA + MEFE (12.5 μM each) or RIBA + MECLO (12.5 μM each). Then, the infected cells were incubated with the compounds for 72 h. The viral load (PFU/ml) was significantly reduced after treatment with the MEFE derivatives compared with the untreated CHIKV-infected cells (One way ANOVA, $P < 0.01$). (B) The figure shows the chemical structures of the test compounds. (C) An ELISA-like cell-based assay was used to evaluate the antiviral activity of the drugs. Vero cells were seeded in 96-well tissue culture plates and infected with CHIKV at an MOI of 1. After removing the viral residues and washing with PBS, the test compounds were added at concentrations that ranged from 0 (control) to 25 μM , while combinations of MECLO + RIBA or MEFE + RIBA were at ratios of 1:1, with final concentrations that were similar to the individual compounds. The infected cells were incubated in the presence of the compounds at 37 °C and 5% CO₂ for 72 h. A viral antigen was detected with a CHIKV antibody and anti-mouse IgG conjugated with alkaline phosphatase. The ELISA experiment results were recorded as the mean of triplicate experiments.

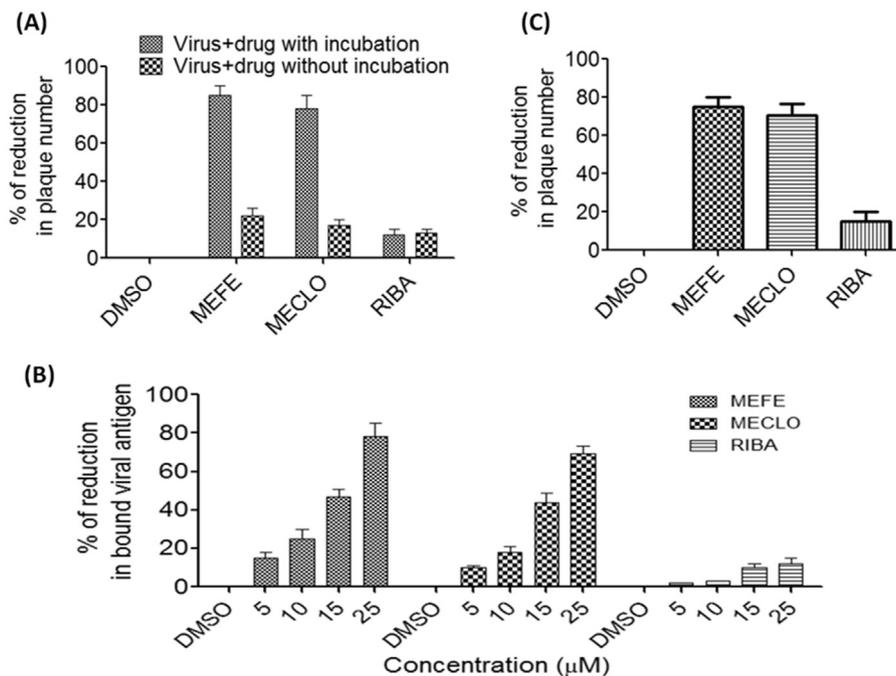


Fig. 2. MEFE and MECLO inactivate viral particles and prevent virus binding and internalizing into Vero cells. (A) The viral inactivation assay. MEFE, MECLO or RIBA (25 μM each) were separately mixed with CHIKV and incubated for 1 h at 37 °C or without incubation and added onto Vero cells. After a 72 h incubation period, viral plaques were stained and counted. (B) Viral attachment analysis using an ELISA-like cell-based assay. Vero cells were seeded into 96-well plates and incubated at 4 °C for 1 h and then inoculated with CHIKV (MOI of 1) in the presence of MEFE, MECLO, RIBA or DMSO (1%), as a control, for another 3 h at 4 °C. The wells were washed and fixed with ice-cold methanol for 15 min at -20 °C and analysed with an ELISA-like assay, as described in the materials and methods section. (C) Viral penetration analysis by plaque reduction assay. Vero cells were incubated at 4 °C for 1 h before inoculation with CHIKV (100 PFU/well) for 3 h at 4 °C. The cells were then treated with 1% DMSO (control), MEFE, MECLO or RIBA (25 μM each) and further incubated for an additional 20 min with the temperature shifted to 37 °C to facilitate viral penetration. At the end of the incubation, the extracellular viruses were inactivated with a citrate buffer (pH 3.0) for 1 min and then the cells were washed with PBS twice before overlaying with medium. After 72 h of incubation at 37 °C, viral plaques were revealed by crystal violet staining and counted. The data were calculated as means and SDs of three independent experiments, with each treatment performed in duplicate.

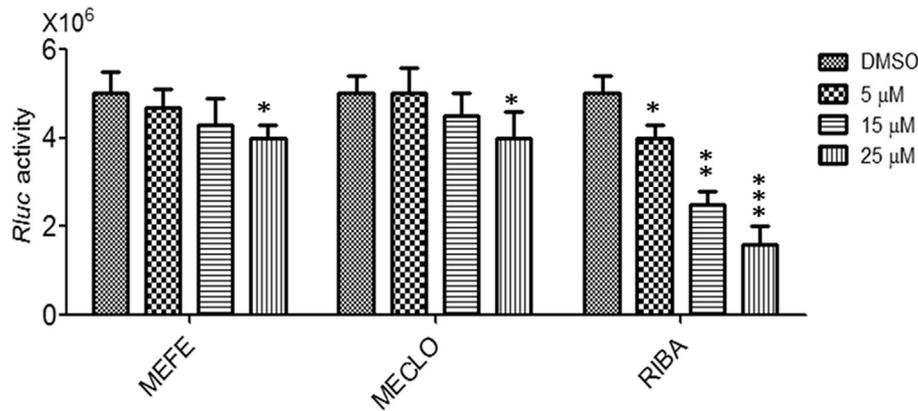


Fig. 3. Evaluation of the anti-CHIKV activity of the test compounds using a CHIKV replicon assay. Huh-7 cells harbouring a CHIKV replicon were seeded into a 96-well plate and treated with the test compounds or DMSO (1%) as a control. After a 48 h incubation, the luminescence signal of the Rluc was measured with a Renilla luciferase assay kit. The MEFE and MECLO treatments both showed a lower inhibitory effect compared with RIBA. The significant effect of these compounds was observed only at 25 μ M ($p < 0.05$). RIBA was the most efficient in reducing the activity of the Rluc reporter in the CHIKV replicon, which was approximately 68% compared with the MEFE and MECLO treatments (20% each) at a similar concentration (25 μ M). Two-way ANOVAs with the Bonferroni post-test were utilized (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

in a 75% and 70% reduction of formed plaque numbers, respectively (compared with DMSO treated cells). Additionally, 20% inhibition was observed when RIBA was used in the same assay. Therefore, it can be postulated that both MEFE and MECLO protected Vero cells from virus penetration. Taken together, our data indicate that MEFE and MECLO impair viral receptor attachment and penetration functions during CHIKV infection.

3.4. Evaluation of the post-entry effect of the compounds using a CHIKV replicon assay

In this assay, the Rluc activity was relative to the viral RNA replication. As expected, RIBA showed the highest inhibition of viral RNA replication, especially at 25 μ M ($p < 0.001$). However, Both MEFE and MECLO showed lower inhibitory effects compared with RIBA. The significant effect of these compounds was observed only at 25 μ M ($p < 0.05$). In other words, RIBA was most efficient in reducing the CHIKV replicon Rluc reporter activity, which was approximately 68% compared with MEFE (20%) and MECLO (20%) treatments at a similar concentration (25 μ M) as shown in Fig. 3.

3.5. Pathological analysis of liver, spleen and kidney tissues from CHIKV-infected mice after test compound treatments

The outcome of an *in vitro* study showed the combinational treatment of MECLO + RIBA or MEFE + RIBA impaired CHIKV replication. MEFE is commercially available, and its synthesis is easier compared with MECLO. Hence, we only tested MEFE in the animal study. The acute toxicity of the test compounds was determined by intraperitoneally administering the animals with low (5 mg/kg) and high doses (50 mg/kg) of the drugs, individually or in combination. The test compounds at these doses showed no signs of toxicity throughout the 24 h observation period. Histological liver, spleen and kidney examinations were similar with the untreated control group after up to 14 days post-treatment (data not shown).

To test the effects of combinational drug treatment, the liver, spleen and kidney weights and morphologies and also the viral titre in blood were analysed. After 7 days post-infection, the CHIKV-infected mice showed liver and spleen hypertrophy, while the kidney sizes were not affected (Fig. S2). To confirm and quantify these effects, the liver and spleen weights were measured. As expected, the CHIKV infection caused increased weight of these

organs (Fig. 4A and B). We observed that treatment with the combinational drugs resulted in a significant reduction of the hypertrophic effects that were caused by CHIKV on the liver and spleen (Fig. 4A and B). Treatment with MEFE-RIBA led to a significant reduction in the liver and spleen weights (Kruskal–Wallis test followed by the Dunn's post-test, $p < 0.01$) compared with the untreated control and RIBA groups ($p < 0.05$). On the contrary, the differences in liver and spleen weights in the RIBA and MEFE groups were insignificant compared with the non-treated controls (Fig. 4A and B). Quantification of viral titres in the blood of the infected mice with a plaque formation assay revealed that compared with the untreated mice, the MEFE-RIBA treated mice had significantly reduced viral amounts in blood (3.45×10^4 PFU and 0.53×10^4 PFU, respectively). Additionally, the RIBA treated group (1.43×10^4 PFU) also exhibited a significant reduction ($p < 0.05$) in viral titres compared with the control group; however, treatment with MEFE alone (2.26×10^4 PFU) did not result in a significant viral titre reduction (Fig. 4C).

4. Discussion

Patients with CHIKV experience high fever (more than 38.9 °C) and chronic joint pain for a range of 4–7 days after virus transmission, wherein it is followed by a second stage of symptoms that include persistent polyarthralgias (Ziegler et al., 2008; Staples et al., 2009). In a portion of cases, CHIKV infection may cause multiple organ dysfunction, which can eventually lead to mortality (Pialoux et al., 2007). However, the current treatment strategies against CHIKV infection depend on alleviating the symptoms that are associated with CHIKV disease because of the unavailability of effective anti-CHIKV drugs or vaccines. Furthermore, treatment with common antiviral drugs, such as RIBA or ACIC, as a monotherapy treatment is not highly effective. In contrast, combining these antivirals with other compounds showed outstanding outcomes (Fuster et al., 2005; Bizollon et al., 2005; Lawitz et al., 2008; Jeulin et al., 2008; Sebastian et al., 2012; Darr et al., 2008). Notably, most of the compounds that are used in combination with RIBA are relatively expensive. As an alternative, drugs that are used to treat musculoskeletal disorders, inflammation and to control pain, such as the NSAIDs, appear as attractive candidates that might alleviate viral symptoms and reduce viral replication, especially in combination with common antiviral drugs, such as RIBA and ACIC.

The current study provides evidence for considerable antiviral

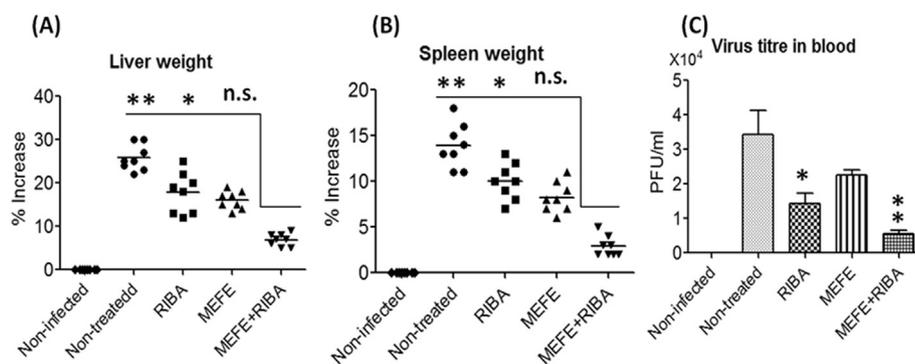


Fig. 4. Treatment with MEFE + RIBA reduces viraemia as well as liver and spleen weights in CHIKV-infected mice. The animal groups (five groups, $n = 8$ each) were intraperitoneally inoculated with 1×10^6 (PFU) of CHIKV. The positive control group was administered PBS, single doses of RIBA (15 mg/kg), MEFE (15 mg/kg) and MEFE + RIBA (15 mg/kg), or PBS were also administered. Mice were observed for 7 days post-infection and sacrificed for viral quantification and pathological analysis. (A) Treatment with the combinational drugs resulted in a considerable reduction in the liver weights compared with RIBA alone ($p < 0.05$) and the untreated control ($p < 0.01$) (B) The hypertrophy of the spleen due to infection was significantly reduced by the combinational treatment compared with the non-treated animals ($p < 0.01$) and the animals that received only RIBA ($p < 0.05$), as shown by the Kruskal–Wallis test followed by the Dunn's post-test. (C) Viral titres in blood of infected mice were quantified with a plaque formation assay (the limit of detection was 10 PFU) after 7 days post-infection. Although RIBA significantly reduced the viral titres, MEFE + RIBA showed a more outstanding reduction compared with the non-treated animals (One Way ANOVA, $P < 0.01$).

activity of the NSAID derivatives in combination with other common antiviral drugs. Our observations showed that the viral yield was significantly reduced in CHIKV-infected cells after treatment with the antiviral drug, RIBA and also with MECLO and MEFE derivatives of the NSAID group. Interestingly, outstanding inhibitory effects against CHIKV were observed following treatment with a combination of MECLO + RIBA or MEFE + RIBA (Fig. 1A).

Additionally, in this study, the analysis of the mode of inhibition suggested that the inhibitory activity of the test compound against CHIKV appears to be at the viral entry stages. The results suggest that the antiviral activity of MEFE and MECLO was due to viral envelop interactions that led to virus inactivation, as demonstrated by incubation of the test compounds with virus particles. Additionally, further antiviral activity was observed against virus binding and internalisation into Vero cells (Fig. 2). Conversely, the test compounds showed limited inhibitory effect toward viral replication compared with RIBA as demonstrated with the CHIKV replicon assay. This fact may lead to the hypotheses that the indirect effects of MEFE and MECLO on certain cellular pathways or enzymes may lead to such virus replication inhibition. These assumptions have been uncovered by the current study and warrant further investigation.

In this study, the EC_{50} against CHIKV was estimated to be 13 μ M for MEFE and 18 μ M for MECLO with a CC_{50} of more than 100 μ M in Vero cells. However, the inhibitory effects of MEFE and MECLO were maximised when administered in combination with RIBA against CHIKV compared with ACIC. Similarly, MEFE showed inhibitory effects against RNA viruses, which was estimated to be 90% at a concentration of 30 μ M (Inglot, 1969). MEFE is widely used as anti-inflammatory drug and therefore, in our *in vivo* study, we selected MEFE to test the antiviral activities in combination with RIBA. The results showed that the MEFE + RIBA treatment led to a significant reduction in virus infection compared with the RIBA alone treatment. Both of these drugs have different mechanisms of action. RIBA seemed to inhibit replication, while MEFE inhibited CHIKV attachment and entry, which may have provided a synergistic (additive) effect. The high dose (10^6 PFU/mice) of CHIKV that was used to examine the ability of the drugs to combat viral infection may lead to longer viral persistence in the serum of the ICR mice (7 days post-infection). Previous studies showed that viral genome was eliminated after 5–6 days in 6- to 10-week-old C57BL/6 strain mice (Yao et al., 2012). It was reported that high a inoculation dose significantly correlated with the viraemia period and level in a

monkey model (Labadie et al., 2010) and also in C57BL/6J mice (Parashar et al., 2013). The results of this study are consistent with previous studies that showed CHIKV persistence in the serum for 7–10 days post-infection (Parashar et al., 2013; Morrison et al., 2011). The MEFE + RIBA treatment led to a considerable reduction in pathological signs (reduced liver and spleen hypertrophy) compared with the individual MEFE or RIBA treatments. The outcomes of the MEFE + RIBA combinational treatment could be due to the accumulated antiviral effects of both compounds in addition to the effect of MEFE as an anti-inflammatory drug. This study could contribute to better understanding of the antiviral activity of MEFE in addition to its feature as an anti-inflammatory drug.

In conclusion, our study demonstrated that the NSAID derivatives, MEFE and MECLO, exhibited significant anti-CHIKV activity that acted by impairing viral replication in Vero cells. The common derivative, MEFE, showed anti-CHIKV activity *in vitro* and *in vivo* in combination with RIBA. Further experimental and clinical studies should be conducted to corroborate their potential utilization for the attenuation of CHIKV symptoms.

Authors' contributions

HAR designed and performed the experiments and drafted the manuscript. HB, TCT, AYA, SO and NNR participated in the experiments. ZQ participated in the data analysis and drafted the manuscript. NSR and RY participated in the design and revised the manuscript. All of the authors approved the final manuscript.

Conflicts of interest

The authors have declared that no competing interests exist.

Acknowledgement

This study was sponsored by the University of Malaya through a UMRG grant (RG309-14AFR and BK043-2014).

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.antiviral.2016.01.006>.

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