



## Suramin treatment reduces chikungunya pathogenesis in mice



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### ABSTRACT

The chikungunya virus (CHIKV), an arthritogenic alphavirus, has caused explosive epidemics involving millions of cases. Globally expanding pandemics involving CHIKV and post-CHIKV rheumatic disorders are increasing public health concerns. However, no antiviral interventions or vaccines to control CHIKV infection have yet been approved. Although suramin has been possess anti-CHIKV activity *in vitro*, whether suramin has anti-CHIKV activity *in vivo* remains unknown. This study aimed to determine whether suramin treatment could ameliorate CHIKV-induced arthritis in a C57BL/6 mice model.

C57BL/6 mice were infected with CHIKVs to evaluate anti-CHIKV activities of suramin in terms of histopathology, viral burden and disease score. Not only did suramin treatment substantially decrease viral loads, but it also significantly ameliorated acute foot lesions in mice. In addition, suramin treatment markedly restores cartilage integrity and reduces the number of IHC positive chondrocyte in mice infected with CHIKV strains 0810bTw and 0706aTw. This *in vivo* study highlights the potential ability of suramin to treat CHIKV infection in clinical settings.

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## 1. Introduction

Chikungunya virus (CHIKV), an enveloped plus-stranded RNA virus, belongs to the genus *alphavirus* of family *Togaviridae*. CHIKV is an arbovirus transmitted by infected *Aedes* mosquito species. In human, symptoms of CHIKV infection include fever, rash, myalgia and persistent incapacitating arthralgia. For example, in 2005, a CHIKV outbreak on Reunion Island caused more than 60% of CHIKV patients to experience arthralgia during the 3 years that followed acute infection (Schilte et al., 2013). Evidence has also suggested that CHIKV infection can lead to rheumatic disorders (Bouquillard and Combe, 2009; Brighton et al., 1983; Javelle et al., 2015; Miner et al., 2015; Zeana et al., 2016), such as chronic arthritis that mimics rheumatoid arthritis (RA), a condition which can last from weeks to years. Moreover, previous research has found that 5.6% of CHIKV patients suffered persistent joint pain and stiffness, and these patients retained very high CHIKV antibody titers three years

after disease onset (Brighton et al., 1983). Finally, two studies have observed CHIKV patients suffering from erosive arthritis, which was induced by post-infection with arthritogenic alphaviruses (Chaaithanya et al., 2014; Manimunda et al., 2010). Subtle genetic change in CHIKV E1-A226V has been associated with an increase of the vector competence for *Ae. albopictus*, but not *Ae. aegypti* (Schuffenecker et al., 2006; Tsetsarkin et al., 2007; Vazeille et al., 2007), and expanding global pandemics of CHIKV are causing an increase in public health concerns. For example, in Latin America, it is estimated that 0.4 million patients will develop post-CHIKV chronic inflammatory rheumatism (Rodriguez-Morales et al., 2015). Currently, there are no approved vaccines and no antiviral interventions to control CHIKV-induced rheumatologic disease. Therefore, advances in antiviral research could greatly benefit the management of CHIKV infections.

Despite significant gaps in the clinical literature, previous studies found that treating CHIKV-infected mice with bindarit, an inhibitor of MCP-1 synthesis, reduced the severity of CHIKV-induced bone loss and musculoskeletal tissue inflammation (Chen et al., 2015; Rulli et al., 2011). Furthermore, synergetic anti-CHIKV effects of Mefenamic acid and ribavirin have been

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observed both *in vitro* and *in vivo* (Rothan et al., 2016); as has anti-CHIKV activity of phosphorodiamidate morpholino oligomers (Lam et al., 2015). T-705 (Favipiravir; 6-fluoro-3-hydroxy-2-pyrazinecarboxamide), a nucleobase mimetic, reduces brain infection and mortality rate by more than 50% in CHIKV-infected AG129 mice (Delang et al., 2014). In CHIKV- or Ross River virus (RRV)-infected mice, pentose polysulfate treatment was reported to decrease acute signs of foot swelling and inflammation (Herrero et al., 2015). Finally, the anti-CHIKV activity of MBZM-N-IBT (1-[(2-methylbenzimidazol-1-yl) methyl]-2-oxo-indolin-3-ylidene] amino] thiourea), a molecular hybrid of 2-methyl benzimidazole and isatin- $\beta$ -thiosemicarbazone, have been found to target the early and late stages of CHIKV infection (Mishra et al., 2016). Suramin is an anti-parasitic drug, used to treat human sleeping sickness caused by trypanosomes and onchocerciasis (McGeary et al., 2008). Suramin has been shown to inhibit CHIKV entry, transmission and replication *in vitro* (Albulescu et al., 2015; Ho et al., 2015). Moreover, there is evidence to suggest that suramin induces immunosuppressive effects by inhibiting CD40/CD154 (Margolles-Clark et al., 2009), purinergic receptor bindings (Liu et al., 2014) and TNF- $\alpha$ /TNF- $\alpha$  receptor binding (Mancini et al., 1999).

A mouse model to evaluate therapeutic agents (Fox et al., 2015; Herrero et al., 2015; Jin et al., 2015; Pal et al., 2013; Rulli et al., 2011) and vaccines (Garcia-Arriaza et al., 2014; Hallengard et al., 2014; Piper et al., 2013; Wang et al., 2011) has been developed to study CHIKV infection. Furthermore, several studies have evaluated the antiviral activities of various drugs by subcutaneously injecting CHIKV into immuno-competent *C57BL/6* mice via footpad induced arthritis (Gardner et al., 2010; Morrison et al., 2011; Rulli et al., 2011), and others have used this mouse model to compare pathogenesis among CHIKV strains (Ashbrook et al., 2014). However, a mouse model has not been used to investigate the effects of suramin on CHIKV infection in detail.

In this study, we exploited the *C57BL/6* mice model to investigate the anti-CHIKV activities of suramin *in vivo*. Specifically, pathogenic profiles of 3 clinical isolates were observed in CHIKV-infected mice. Treatment with suramin was found to reduce viral burdens, alleviate CHIKV-induced mice foot swelling and decrease histopathologic lesions. Taken together, our findings suggest that suramin has potential protective effects against CHIKV infection *in vivo*.

## 2. Materials and methods

### 2.1. Viruses, cells and chemical

BHK-21 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 5% heat inactivated fetal bovine serum (FBS) and antibiotics under 5% CO<sub>2</sub> at 37 °C. C6/36 cells were cultured in RPMI-1640 medium with 10% heat inactivated FBS and antibiotics

under 5% CO<sub>2</sub> at 28 °C. CHIKV strains 0611aTw (Singapore/0611aTw/2006/FJ807896), 0810bTw (Malaysia/0810bTw/2008/FJ807899) and 0706aTw (Indonesia/0706aTw/2007/FJ807897) were amplified and titers were determined by conducting a plaque assay in BHK-21 cells (Ho et al., 2015). Suramin was purchased from Sigma-Aldrich (catalog #S2671).

### 2.2. Ethics statement

All animals were handled in strict accordance with good animal practice as defined by the Council of Agriculture, Executive Yuan, Taiwan R.O.C. Protocol involving animals were approved by the Institutional Animal Care and Use Committee of the Institute of Preventive Medicine, National Defense Medical Center. Taiwanese's regulations classify CHIKV is as a bio-agent in Risk Group 3. All studies with viable CHIKV were performed in certified BSL-3 laboratories. Biosafety protocols used by this study were approved by the Institutional Biosafety Committees of the Institute of Preventive Medicine, National Defense Medical Center.

### 2.3. CHIKV challenge study

To investigate the anti-CHIKV activities of suramin *in vivo*, *C57BL/6JNarl* (B6) mice of specific pathogen-free condition were purchased from the National Laboratory Animal Center (P.O. Box 1-86 Nankang, Taipei City 11529, Taiwan R.O.C.). All mice were female and aged 4 weeks. There were 35, 40 and 5 mice ( $n = 5$  per group) used for testing anti-CHIKV activities of suramin in viral strains, dose/time related and foot swelling over time assays, respectively. A schedule detailing incubation with CHIKV, treatment with suramin and examination of mice is provided in Fig. 1. Suramin was diluted in normal saline, and indicated doses were administered at 4 h pre-infection, 1 day post-infection or/and 3 day post-infection (dpi) via the intraperitoneal (ip) route. Specifically, each dose comprised 0, 0.25, 0.5, 1 or 2 mg suramin. Mice were subcutaneously (s.c.) inoculated with 50  $\mu$ l CHIKV ( $10^5$  pfu) via the ventral side of the right hind foot, towards the ankle. Submandibular blood was collected at 2 dpi for viremia analysis. The height and width of the metatarsal area of hind feet were measured using Kincome digital verniercalipers at 7 dpi. After mice were euthanized via an overdose of isoflurane anesthesia followed by cervical dislocation. Hind feet from scarified mice were collected for histopathologic analysis at 7 dpi.

### 2.4. Histopathology and immunohistochemistry (IHC)

Hind feet were fixed in 10% buffered formalin (Electron Microscopy Sciences), decalcified with 15% EDTA in 0.1% phosphate buffer over 10 days and embedded in paraffin wax. We cut 3- $\mu$ m-thick sections from the wax and stained these sections with hematoxylin-eosin or Safranin-O/Fast Green dye. Histological

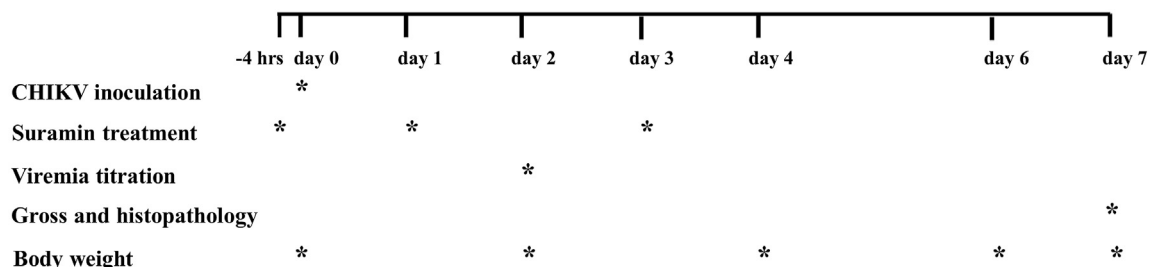


Fig. 1. Schedule detailing CHIKV inoculation, suramin treatment and examination of *C57BL/6* mice.

evaluation was conducted according to the following a scoring system (Romans et al., 2002). 0 = no inflammation, 1 = minimal inflammatory infiltration, 2 = mild infiltration, 3 = moderate infiltration with moderate edema, 4 = marked infiltration with marked edema, 5 = severe infiltration with edema ( $n = 5$  feet per group). For immunohistochemistry analysis, the 3- $\mu\text{m}$ -thick sections were deparaffinized with xylene and rehydrated in serial dilutions of ethanol. Antigens were retrieved by immersing tissue sections into epitope retrieval buffer. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide solution, and slides were incubated with protein blocking buffer for 10 min. Subsequently, tissue sections were incubated with rabbit anti-CHIKV E2 sera (1/1000) (Kuo et al., 2011) at room temperature for 30 min, washed in TBST at pH 7.0, and developed using the Polink-2 HRP Plus Rabbit DAB Detection System (Golden Bridge International, Inc.) according to manufacturer's protocol. All pictures (six pictures from 2 feet per group) were taken under the same lighting and magnification parameters with an Olympus BX43 microscope. The intensity of CHIKV E2 signals was measured using ImageJ software. For this, color deconvolution was used to separate DAB stains (CHIKV E2 signals) from hematoxylin stain (whole tissue area). Specifically, each picture was converted to grayscale and adjusted using the Thresholding method, and the areas of DAB and hematoxylin stains were measured (Analyze/Measure). E2 expression score of CHIKV was calculated by dividing the total tissue area by the positively stained area (Safadi et al., 2010).

### 2.5. Measurements of viremia

Sera from submandibular blood of CHIKV-infected or mock mice (i.e. mice infected with PBS containing 10% culture medium) were stored at  $-70^\circ\text{C}$ . In a 96-well plate,  $3 \times 10^5$  C6/36 cells per well were inoculated with 100  $\mu\text{l}$  medium containing a serial 10-fold diluted blood sample in duplicate. At 3 dpi, fixed cells were stained with rabbit anti-CHIKV E2 serum (1/100) (49) and Alex 488 goat anti-rabbit IgG (1/500) (Invitrogen, Molecular Probes, Carlsbad, CA). Viral loads were expressed as CCID<sub>50</sub>/ml of serum (Gardner et al., 2010).

### 2.6. Statistical analysis

All statistical analyses were performed using GraphPad Prism version 6.0.1 software. Differences between the mock-treated group and the suramin-treated group were assessed using the student's *t*-test. P values are mean  $\pm$  SD for  $n = 5$  or 6.

## 3. Results

### 3.1. Treatment with suramin decreases viral burden and foot swelling in CHIKV-infected mice

To assess the anti-CHIKV activity of suramin *in vivo*, mice were infected with one of the following CHIKV strains: 0611aTw, 0810bTw or 0706aTw. Several previous studies have found that the viremia peak of CHIKV-infected mice is at 2 dpi (Garcia-Arriaza et al., 2014; Gardner et al., 2010; Poo et al., 2014a, 2014b; Wang et al., 2011). Infected mice were treated with either 2 mg suramin or mock at 4 h pre-infection, 1 dpi and 3 dpi (Fig. 1). In the current study, viral loads in sera of mock-treated mice infected with the 0810bTw, 0611aTw and 0706aTw CHIKV strains at the 2 dpi peak were 5.8, 3 and 4.2 Log<sub>10</sub> CCID<sub>50</sub>/ml, respectively (Fig. 2A). To parallel, viral titers of suramin-treated sera after received two doses (pre 4 h and 1 dpi) were 5, 2.5 and 3.8 Log<sub>10</sub> CCID<sub>50</sub>/ml for 0810bTw, 0611aTw and 0706aTw strains, respectively. Therefore, suramin treatment significantly decreased in viral loads in 0810bTw-

infected mice.

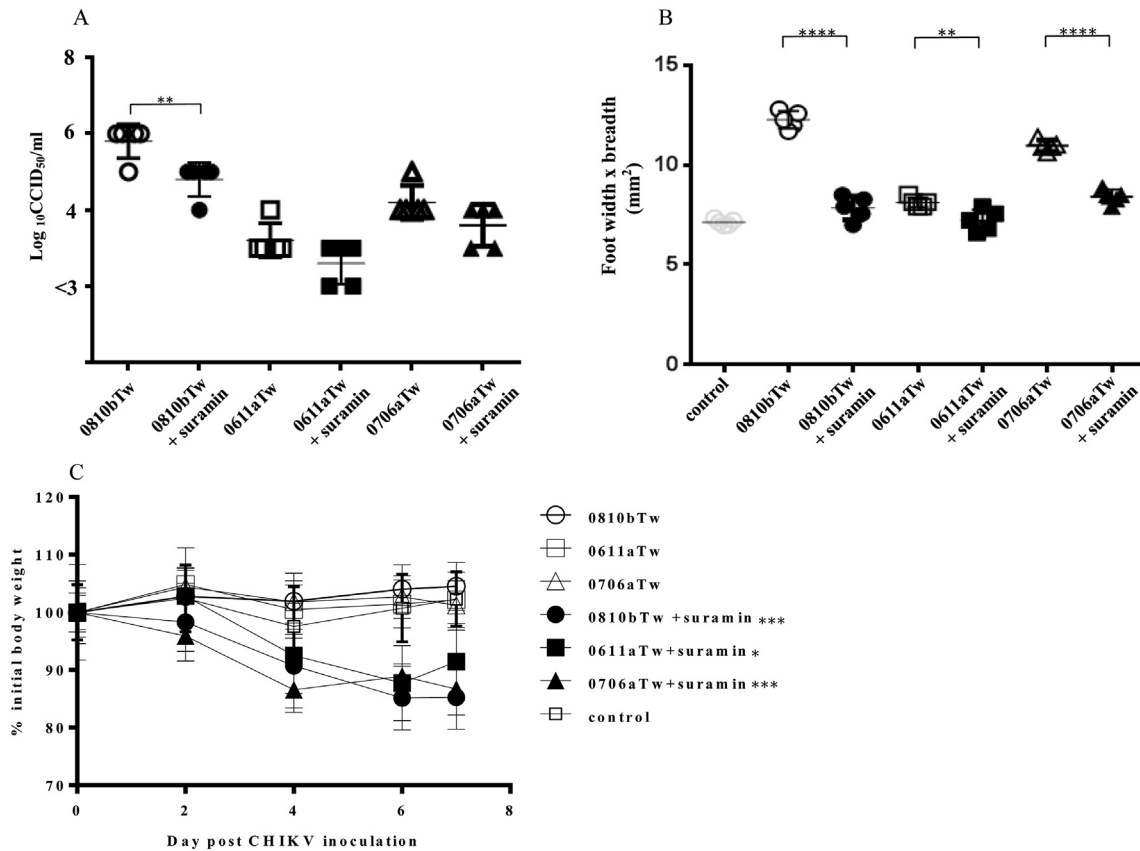
In order to determine the peak of foot swelling induced by CHIKV infection, we quantified foot swelling over time in 0810bTw-infected mice (Fig. S1). Maximum foot swelling occurred at 7 dpi, which corresponds to results obtained by previous studies (Gardner et al., 2010; Poo et al., 2014a). To further characterize the therapeutic effects of suramin treatment on CHIKV infections, foot swelling and histopathologic lesions at peak disease (7 dpi) were examined for both suramin-treated and mock-treated mice. As shown in Fig. 2B, paw volumes of mock-treated mice infected with 0810bTw, 0611aTw and 0706aTw were  $12.28 \pm 0.2$ ,  $8.13 \pm 0.11$  and  $10.98 \pm 0.12$  mm<sup>2</sup>, respectively. Corresponding to the result of viremia, strains 0810bTw and 0706aTw caused swelling and edema which were much more severe than that associated with the 0611aTw strain. Interestingly, paw volumes of suramin-treated mice infected with strains 0810bTw ( $7.85 \pm 0.27$  mm<sup>2</sup>), 0611aTw ( $7.22 \pm 0.24$  mm<sup>2</sup>) and 0706aTw ( $8.42 \pm 0.16$  mm<sup>2</sup>) decreased by 36, 11 and 23% compared to their mock-treated counterparts. Furthermore, suramin treatment decreased the size of hind feet in CHIKV-infected mice to nearly that of control ( $7.13 \pm 0.07$  mm<sup>2</sup>) at 7 dpi. No detectable weight loss was observed in mock-treated mice and control mice (Fig. 2C), but suramin treatment resulted in significant weight loss. However, significant weight gains in suramin-treated mice have been observed at 9 dpi (data not shown); therefore, the weight loss effect of suramin treatment was temporary. Taken altogether, our results suggest that suramin treatment substantially reduces viral burden and also alleviates CHIKV-induced foot swelling in mice.

### 3.2. Treatment with suramin alleviates musculoskeletal lesions in CHIKV-infected mice

To confirm the effects of suramin treatment in foot swelling in CHIKV-infected mice, histological analysis was conducted to examine the histopathological profile in infected feet. Microscopic results at maximum foot swelling (7 dpi) showed that the feet of 0706aTw- and 0810bTw-infected mice had extensive acute lesions including inflammatory infiltrates, subcutaneous edemas, fibrinous exudates and periostitis (Fig. 3A). Conversely, infection with the 0611aTw strain only induced mild inflammatory infiltrates. Control mice did not show any inflammatory infiltrates. Interestingly, suramin treatment led to a marked reduction in acute foot lesions of mice infected with all three strains of CHIKV compared to mock-treated mice, and histopathological scores revealed that suramin treatment led to a statistically significant reduction in inflammatory infiltrates (Fig. 3C).

To further confirm the effects of suramin treatment on viral infection, IHC was performed to detect the expression of viral antigen (E2 glycoprotein) in infected tissues. The specificity of IHC is illustrated in Fig. S2. In the feet of CHIKV-infected mice, IHC signals were mainly observed in macrophages, epithelial cells, bone cells, chondrocytes, periosteum and muscle cells. Similar to the histological inflammation scores, a more intense E2 signal was detected in mock-treated mice infected with either 0706aTw or 0810bTw than in mock-treated mice infected with 0611aTw (Fig. 3B and D). However, suramin treatment uniformly prohibited the expression of E2 glycoprotein in hind foot tissues of infected mice (Fig. 3B and D). Therefore, the IHC analysis confirms that suramin treatment can significantly inhibit CHIKV replication *in vivo*.

In mice models, RRV and CHIKV infections cause the destruction of cartilage including through the depletion of matrix proteoglycan (Herrero et al., 2015; Poo et al., 2014a). Thus, we sought to further elucidate the therapeutic effects of suramin in CHIKV-infected mice by evaluating cartilage integrity. At 7 dpi, IHC and histological evaluations of joint sections stained with Safranin O-fast green dye



**Fig. 2.** Evaluation of anti-CHIKV activity of suramin in mice. Mice were inoculated with the following CHIKV strains via the right hind foot: 0611aTw, 0810bTw or 0706aTw. For all CHIKV strains, mice were subcutaneously (s.c.) inoculated with 50  $\mu$ l CHIKV ( $10^5$  pfu) via the ventral side of the right hind foot. To establish mock treatment groups, some CHIKV-infected mice were administered with 100  $\mu$ l normal saline (0810bTw, 0611aTw and 0706aTw). Treatment groups comprised CHIKV-infected mice which were administered the indicated dose (0810bTw + suramin, 0611aTw + suramin and 0706aTw + suramin) at 4-h pre-infection, 1 dpi and/or 3 dpi by the ip route. The height and width of the metatarsal area of right hind feet were measured using Kincrome digital verniercalipers at 7 dpi. For control mice, 50  $\mu$ l diluted medium (1/10) was injected into right hind feet, and treatment involved administering 100  $\mu$ l normal saline via the ip route at 4-h pre-infection, 1 dpi and 3 dpi. P values were determined using the Student's *t*-test. A. Effects of suramin treatment on viral burdens. Viremia measurements for CHIKV-infected mice were determined using the CCID<sub>50</sub> method at 2 dpi. Each symbol represents an individual sample. Bars show the mean  $\pm$  SEM. B. Effect of suramin treatment on CHIKV-induced foot swelling. Each symbol represents an individual sample. Bars show the mean  $\pm$  SEM. C. Average weight of CHIKV-infected mice in mock-treated and suramin-treated groups. Body weight was measured at 0, 2, 4, 6 and 7 dpi. Values denote average weight  $\pm$  SD for; \**p* < 0.05, \*\**p* < 0.01 and \*\*\**p* < 0.001 compared to the control group at 7 dpi.

revealed that CHIKV strains 0810bTw and 0706aTw led to more severe cartilage destruction (Fig. 4A) and a great abundance of E2-positive chondrocytes than did the 0611aTw strain (Fig. 4B). However, suramin treatment markedly restored cartilage integrity and reduced the number of IHC positive chondrocyte in mice infected with 0810bTw and 0706aTw.

Taken together, the results described in this section clearly demonstrate that suramin treatment decreases the infectivity of CHIKV and ameliorates CHIKV-induced arthritis in C57BL/6 mice.

### 3.3. Dose- and time-related evaluations of suramin treatment

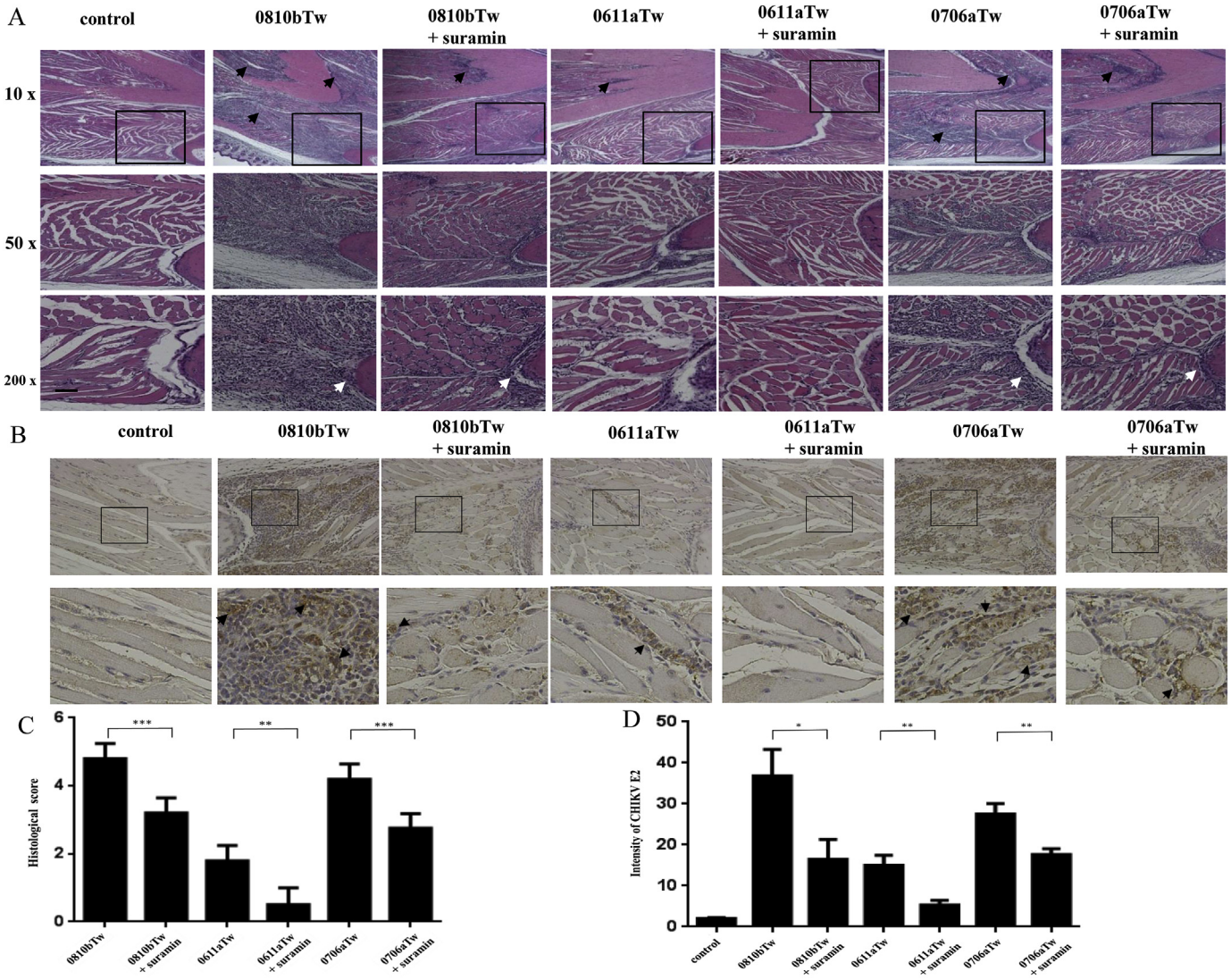
In this study, the most significant therapeutic effects of suramin treatment were observed in 0810bTw-infected mice. We therefore used the CHIKV0810bTw strain to perform dose- and time-related assays. For the dose-related assay, infected mice were treated with three-doses of two-fold serial diluted suramin (2, 1, 0.5 or 0.25 mg) at 4 h pre-infection, 1 dpi and 3 dpi. For the time-related assay, infected mice were administered a single dose of 2 mg suramin at 4 h pre-infection (pre 2 mg), or post-treated with two doses of 2 mg suramin at 1 dpi and 3 dpi (post 2 mg). We observed that suramin variably decreased viremia in dose- and time-related assays (Fig. 5A). Foot swelling under treatment with 2 mg

( $7.85 \pm 0.27$  mm<sup>2</sup>), 1 mg ( $8.59 \pm 0.28$  mm<sup>2</sup>), 0.5 mg ( $9.83 \pm 0.24$  mm<sup>2</sup>), 0.25 mg ( $11.05 \pm 0.3$  mm<sup>2</sup>), pre 2 mg ( $9.31 \pm 0.16$  mm<sup>2</sup>) and post 2 mg suramin ( $8.94 \pm 0.13$  mm<sup>2</sup>) was reduced by 34, 28, 18, 8, 22 and 25%, respectively, compared to mock-treated mice ( $11.98 \pm 0.36$  mm<sup>2</sup>) (Fig. 5B). Interestingly, a single dose of 2 mg suramin treatment at 4 h pre-infection (pre 2 mg) led to a markedly reduced viral burden and significantly decreased foot swelling. Results from average weight monitoring (Fig. 5C), showed dose-dependent weight loss in suramin-treated mice. Notably, all dose- and time-dependent treatments of suramin substantially reduced viral burden and disease score in CHIKV-infected mice.

## 4. Discussion

Since the 2005 CHIKV outbreak that occurred on Reunion Island, CHIKV disease incidence and persistent CHIKV-induced RA-like symptom have been a burden on public health. Due to a lack of specific anti-CHIKV drugs, the nosological approach is presently the only treatment option for post-CHIKV rheumatic disorders. Currently, alphaviral arthritides can be relieved with analgesics and/or nonsteroidal anti-inflammatory drugs (NSAIDs) (Jaffar-Bandjee et al., 2010; Mylonas et al., 2002; Suhrbier and La Linn,



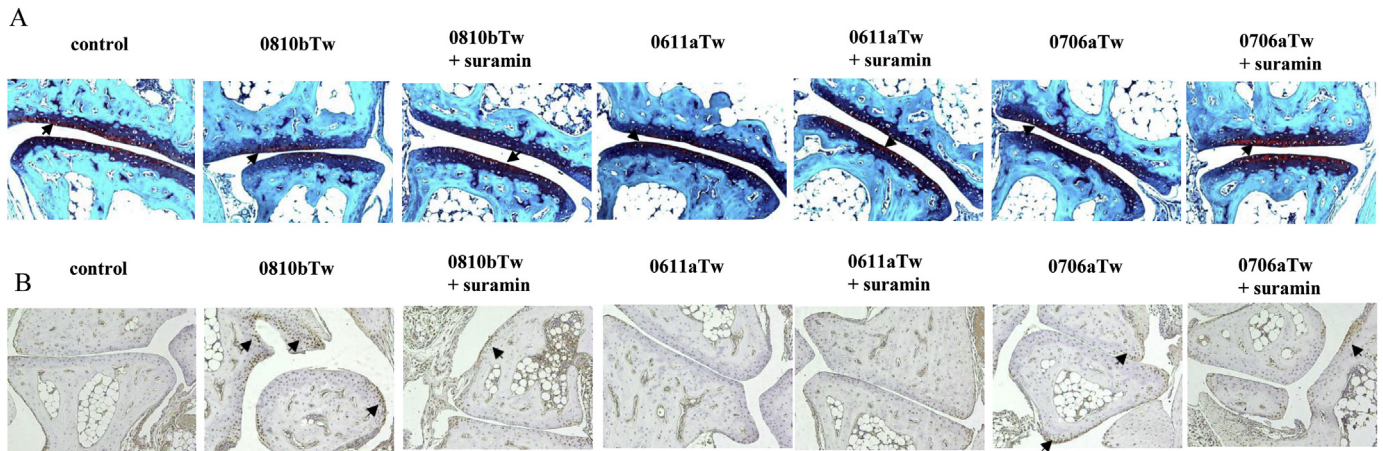


**Fig. 3.** Effect of suramin treatment on CHIKV-induced histopathological profiles. **A.** Effect of suramin treatment on CHIKV-induced inflammation. Right hind feet collected at 7 dpi were fixed, decalcified and stained with hematoxylin-eosin. Boxed areas in upper panels represent the same tissue as that shown in the bottom panels, albeit at a higher resolution ( $\times 200$  versus  $50\times$  magnifications). The foci of myositis are delineated by black arrows. Periostitis in CHIKV-infected mice is indicated by white arrows with inflammatory cells present in the tendon capsule. (Bars:  $100\ \mu\text{m}$ ). **B.** Effects of suramin treatment on the expression of viral E2 antigen in the feet of CHIKV-infected mice. Effects were determined by conducting an immunohistochemical analysis of E2 glycoprotein in feet from suramin-treated or mock-treated mice at 7 dpi. Boxed areas in upper panels present the same tissue section as that shown in bottom panels, albeit at a higher resolution ( $\times 800$  magnification). The foci of E2 glycoprotein are indicated by black arrows. **C.** Histological evaluation of suramin treatment on CHIKV-induced inflammation. The evaluation was conducted according to the following scoring system: 0 = no inflammation, 1 = minimal inflammatory infiltration, 2 = mild infiltration, 3 = moderate infiltration with moderate edema, 4 = marked infiltration with marked edema, 5 = severe infiltration with edema. Values present the mean  $\pm$  SEM. P values were determined using the Student's *t*-test. **D.** Intensity of CHIKV E2 signals in the feet of CHIKV-infected mice. Values presented in the graphs denote mean  $\pm$  SEM ( $n = 6$ ).

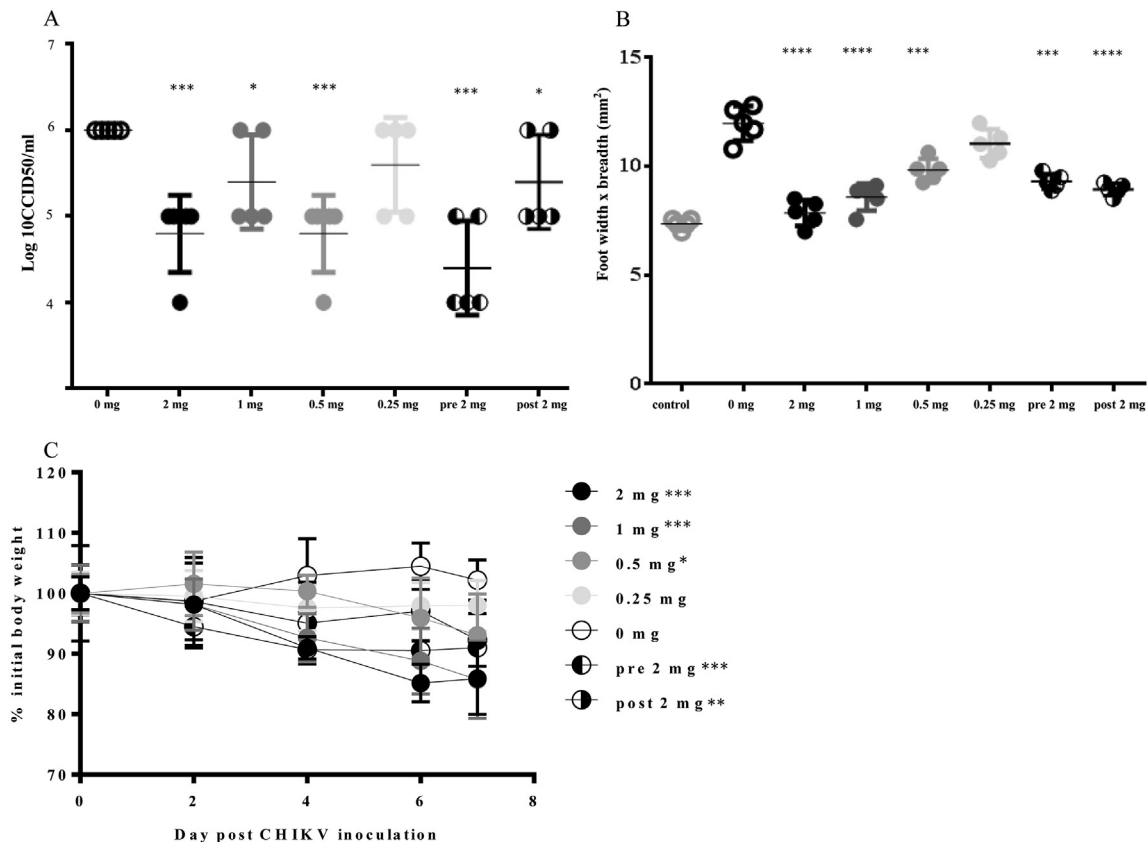
2004; Toivanen, 2008); and treating CHIKV-induced RA-like arthritis with NSAIDs alone or conjugation with steroid leads to a positive clinical response (Rosario et al., 2015). CHIKV-induced RA-like arthritis can also be efficiently treated with methotrexate of disease-modifying anti-rheumatic drug (DMARD) therapy (Chopra et al., 2008; Javelle et al., 2015). However, the immunosuppressive activities of the aforementioned drugs should be considered during the acute phase of CHIKV infection.

Suramin shows a broad-spectrum of antiviral activities *in vitro*. Our study has found that suramin blocks the entry and egress of CHIKV infection (Ho et al., 2015). In this study, we employed a *C57BL/6* mouse model to investigate the pathogenesis of CHIKV infections as well as the efficacy of suramin treatment against this disease *in vivo*. Results showed that viral loads of CHIKV strains

0810bTw, 0611aTw and 0706aTw strains during peak viremia (2 dpi) were 5.8, 3 and 4.2  $\text{Log}_{10}$  CCID<sub>50</sub>/ml, respectively (Fig. 2A). Infection with the three strains of CHIKV also induced (with vary degrees of severity) foot swelling, myositis, synovitis, cartilage destruction, periostitis and perivascularitis (Figs. 2B and 3A, C). Gross and histological results were correlated with viral burden, as evidenced by findings from virus titration and IHC (Figs. 2A and 3B, D). In humans, CHIKV viral antigens have previously been detected in arthritic capsules, skeletal muscle and dermis (Couderc et al., 2008). Furthermore, human muscle satellite cells have been identified as the target of CHIKV (Ozden et al., 2007). In this study, CHIKV E2 was detected in inflammatory infiltrates, epithelial cells, bone cells, chondrocytes, periosteum and muscle cells in CHIKV-infected feet (Figs. 3B and 4B), which corresponds to



**Fig. 4.** Effect of suramin treatment on CHIKV-induced cartilage damage. A. Safranin-O/Fast Green staining to evaluate CHIKV-induced cartilage destruction in mock-treated or suramin-treated mice at 7 dpi. Proteoglycans (in red) are denoted by black arrows. B. Immunohistochemical analysis to detect E2 glycoprotein expression in cartilage from mock-treated or suramin-treated mice at 7 dpi. The foci of E2 glycoprotein are indicated by black arrows. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 5.** Dose- and time-related evaluations of suramin treatment in CHIKV-infected mice. Mice were inoculated with  $10^5$  pfu of CHIKV strain 0810bTw via the right hind foot. For the dose-related assay, CHIKV infected mice were treated with three doses of 0, 2, 1, 0.5 or 0.25 mg of suramin at 4 h pre-infection, 1 dpi and 3 dpi. For time-related assay, we compared the effectiveness of two treatment regimens: pre-treatment (at 4 h pre-infection; pre 2 mg) and post-treatment (at 1 dpi and 3 dpi; post 2 mg). A \* symbol indicates that differences between mock-group and treatment-group mice were significant. A. Dose- and time-related evaluations of suramin treatment on viral burdens. Viremia measurements of CHIKV-infected mice were determined using the CCID<sub>50</sub> method at 2 dpi. B. Dose- and time-related evaluations of suramin treatment on CHIKV-induced foot swelling. The height and width of the metatarsal area of right hind feet were measured using Kincome digital verniercalipers at 7 dpi. Control mice were injected with 50  $\mu$ l diluted medium (1/10) via the right hind foot, and were treated with 100  $\mu$ l normal saline at 4 h pre-infection, 1 dpi and 3 dpi via the ip route. C. Average weight of CHIKV-infected mice in dose- and time-related evaluations. Body weight was measured at 0, 2, 4, 6 and 7 dpi. Values denote average weight  $\pm$  SD; \*p < 0.05, \*\*p < 0.01 and \*\*\*p < 0.001 compared to the group that received 0 mg at 7 dpi.

characteristics of cell and tissue tropisms associated with CHIKV infection in humans. Analysis of cartilage damage showed that infection with the 0810bTw strain induces obvious proteoglycan

depletion and abundant E2-positive chondrocytes. Therefore, in mice, the pathogenic profiles of CHIKV strain 0810bTw, 0611aTw and 0706aTw were correlated with their virulence.



Suramin treatment reduced viral loads in 0810bTw-, 0611aTw- and 0706aTw-infected mice which might due to suramin treatment to block CHIKV entry and egress (Figs. 2A and 5A) (Ho et al., 2015). However, 0810bTw was the most sensitive strain to suramin treatment among the 3 CHIKV isolates *in vivo* which corresponded to their EC<sub>50</sub> activities *in vitro* (Ho et al., 2015). Suramin treatment also ameliorated foot swelling and reduced inflammatory infiltration, which corresponded to reduced viremia and viral antigen expression in infected tissues. In addition, results from Safranin-O/Fast Green staining and IHC analysis showed that suramin treatment restored the integrity of foot cartilage in 0810bTw-infected mice (Fig. 4A and B). Furthermore, we found that suramin induces a dose-dependent reduction in foot swelling in CHIKV 0810bTw-infected mice (Fig. 5A–B), and the vary degrees of decreased viremia that was detected in suramin-treated mice further confirmed therapeutic effects of this drug. In the time-related assay, a single dose of 2 mg suramin (at 4 h pre-infection) or double doses of 2 mg suramin (at 1 dpi and 3 dpi) significantly decreased disease score and viremia compared to mock-treated mice. Due to the limitations of viremia determination at 7 dpi (feet swelling measurement), we were only able to detect viremia of three strains at peak (i.e. 2 dpi; when mice had only received 2 doses of suramin), which may not fully reflect the anti-CHIKV activity of suramin. Nevertheless, the expressions of the viral E2 protein (which provides further evidence of viral load), detected by IHC at 7 dpi (after mice received 3 doses of suramin), was significantly reduced. This results showing reduced foot swelling, inflammation and cartilage damage, indicate that suramin has therapeutic effects on CHIKV-infected mice. Dose-dependent weight loss also was observed in dose- and time-related evaluations of suramin treatment. Importantly, a single pre-treatment of 2 mg suramin at 4 h pre-infection optimized therapeutic effects and weight maintenance. Taken together, our findings clearly demonstrate the anti-CHIKV activities of suramin *in vivo*. The immunosuppressive effects of suramin have also been found to alleviate collagen induced arthritis in rat (Sahu et al., 2012) and acute rejection of lung allograft in mice (Liu et al., 2014). Therefore, the immunosuppressive effects of suramin may have partly contributed to the relief CHIKV-induced arthritis in mice.

The highest EC<sub>50</sub> of strain 0611aTw in U2OS cell was 71.5 μM (59.6 + 11.9 μM; 102 μg/ml) (Ho et al., 2015), and the average body volume was 19 ml (average body weight/average body density = 20 g/1.05) (Amaro et al., 2014). In order to test the maximum anti-CHIKV activity of suramin *in vivo*, a 2 mg dose (2 mg suramin/19 ml/mice = 105 μg/ml) was first administered. Some evidence has suggested that a 2 mg dose is toxic in mice (Yoneda et al., 1995); however, our results showed that 2 mg suramin significantly reduces E2 intensity, pathological scores, and transient weight loss. In addition, viral load was significantly reduced after mice were treated with two doses of 0.5 mg suramin (Fig. 5A; 30 mg/kg). A higher dose of 50 mg suramin/kg has previously been used to evaluate the anti-enterovirus 71 activity of suramin in mice (Ren et al., 2014). In the time-related assay, we found that pre-treating mice with a 2 mg dose was most effective at reducing viral load. Therefore, findings from this study suggest that suramin may have both prophylactic and therapeutic advantages for CHIKV treatment. Future studies could benefit CHIKV research by investigating the dose dependent effects of suramin pretreatment. Identifying the minimal pre-treatment dose should help further elucidate the prophylactic effects of suramin.

In conclusion, our findings clearly demonstrate that suramin treatment decreases viral burden and helps mitigate acute disease symptoms in CHIKV-infected mice. This preclinical study provides evidence to support clinical trials which investigate the feasibility of using suramin to treat chikungunya Fever.

## Conflicts of interest

The authors have no conflicting financial interests.

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## Appendix ASupplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.antiviral.2016.07.025>.

## References

- Albulescu, I.C., van Hoolwerff, M., Wolters, L.A., Bottaro, E., Nastruzzi, C., Yang, S.C., Tsay, S.C., Hwu, J.R., Snijder, E.J., van Hemert, M.J., 2015. Suramin inhibits chikungunya virus replication through multiple mechanisms. *Antivir. Res.* 121, 39–46.
- Amaro, C.A., Gonzalez-Cortazar, M., Herrera-Ruiz, M., Roman-Ramos, R., Aguilar-Santamaria, L., Tortoriello, J., Jimenez-Ferrer, E., 2014. Hypoglycemic and hypotensive activity of a root extract of *Smilax aristolochifolia*, standardized on N-trans-feruloyl-tyramine. *Molecules* 19, 11366–11384.
- Ashbrook, A.W., Burrack, K.S., Silva, L.A., Montgomery, S.A., Heise, M.T., Morrison, T.E., Dermody, T.S., 2014. Residue 82 of the chikungunya virus E2 attachment protein modulates viral dissemination and arthritis in mice. *J. Virol.* 88, 12180–12192.
- Bouquillard, E., Combe, B., 2009. A report of 21 cases of rheumatoid arthritis following chikungunya fever. A mean follow-up of two years. *Jt. Bone Spine Rev. Rhum.* 76, 654–657.
- Brighton, S.W., Prozesky, O.W., de la Harpe, A.L., 1983. Chikungunya virus infection. A retrospective study of 107 cases. *South Afr. Med. J. Suid Afrik. tydskr. vir geneesk.* 63, 313–315.
- Chaitanya, I.K., Muruganandam, N., Raghuraj, U., Sugunan, A.P., Rajesh, R., Anwesh, M., Rai, S.K., Vijayachari, P., 2014. Chronic inflammatory arthritis with persisting bony erosions in patients following chikungunya infection. *Indian J. Med. Res.* 140, 142–145.
- Chen, W., Foo, S.S., Taylor, A., Lulla, A., Merits, A., Hueston, L., Forwood, M.R., Walsh, N.C., Sims, N.A., Herrero, L.J., Mahalingam, S., 2015. Bindarit, an inhibitor of monocyte chemotactic protein synthesis, protects against bone loss induced by chikungunya virus infection. *J. Virol.* 89, 581–593.
- Chopra, A., Anuradha, V., Lagoo-Joshi, V., Kunjir, V., Salvi, S., Saluja, M., 2008. Chikungunya virus aches and pains: an emerging challenge. *Arthritis Rheum.* 58, 2921–2922.
- Couderc, T., Chretien, F., Schilte, C., Disson, O., Brigitte, M., Guivel-Benhassine, F., Touret, Y., Barau, G., Cayet, N., Schuffenecker, I., Despres, P., Arenzana-Seisdedos, F., Michault, A., Albert, M.L., Lecuit, M., 2008. A mouse model for chikungunya: young age and inefficient type-I interferon signaling are risk factors for severe disease. *PLoS Pathog.* 4, e29.
- Delang, L., Segura Guerrero, N., Tas, A., Querat, G., Pastorino, B., Froeyen, M., Dallmeier, K., Jochmans, D., Herdewijn, P., Bello, F., Snijder, E.J., de Lamballerie, X., Martina, B., Neyts, J., van Hemert, M.J., Leyssen, P., 2014. Mutations in the chikungunya virus non-structural proteins cause resistance to favipiravir (T-705), a broad-spectrum antiviral. *J. Antimicrob. Chemother.* 69, 2770–2784.
- Fox, J.M., Long, F., Edeling, M.A., Lin, H., van Duijl-Richter, M.K., Fong, R.H., Kahle, K.M., Smit, J.M., Jin, J., Simmons, G., Doranz, B.J., Crowe Jr., J.E., Fremont, D.H., Rossmann, M.G., Diamond, M.S., 2015. Broadly neutralizing alphavirus antibodies bind an epitope on E2 and inhibit entry and egress. *Cell* 163, 1095–1107.
- Garcia-Arriaza, J., Cepeda, V., Hallengard, D., Sorzano, C.O., Kummerer, B.M., Liljestrom, P., Esteban, M., 2014. A novel poxvirus-based vaccine, MVA-CHIKV, is highly immunogenic and protects mice against chikungunya infection. *J. Virol.* 88, 3527–3547.
- Gardner, J., Anraku, I., Le, T.T., Larcher, T., Major, L., Roques, P., Schroder, W.A., Higgs, S., Suhrbier, A., 2010. Chikungunya virus arthritis in adult wild-type mice. *J. Virol.* 84, 8021–8032.
- Hallengard, D., Kakoulidou, M., Lulla, A., Kummerer, B.M., Johansson, D.X., Mutso, M., Lulla, V., Fazakerley, J.K., Roques, P., Le Grand, R., Merits, A., Liljestrom, P., 2014. Novel attenuated chikungunya vaccine candidates elicit protective immunity in C57BL/6 mice. *J. Virol.* 88, 2858–2866.
- Herrero, L.J., Foo, S.S., Sheng, K.C., Chen, W., Forwood, M.R., Bucala, R.,

- Mahalingam, S., 2015. Pentosan polysulfate: a novel glycosaminoglycan-like molecule for effective treatment of alphavirus-induced cartilage destruction and inflammatory disease. *J. Virol.* 89, 8063–8076.
- Ho, Y.J., Wang, Y.M., Lu, J.W., Wu, T.Y., Lin, L.I., Kuo, S.C., Lin, C.C., 2015. Suramin inhibits chikungunya virus entry and transmission. *PLoS One* 10, e0133511.
- Jaffar-Bandjee, M.C., Ramful, D., Gauzere, B.A., Hoarau, J.J., Krejbich-Trotot, P., Robin, S., Ribera, A., Selambarom, J., Gasque, P., 2010. Emergence and clinical insights into the pathology of chikungunya virus infection. *Expert Rev. Anti Infect. Ther.* 8, 987–996.
- Javelle, E., Ribera, A., Degasne, I., Gauzere, B.A., Marimoutou, C., Simon, F., 2015. Specific management of post-chikungunya rheumatic disorders: a retrospective study of 159 cases in Reunion Island from 2006–2012. *PLoS Negl. Trop. Dis.* 9, e0003603.
- Jin, J., Liss, N.M., Chen, D.H., Liao, M., Fox, J.M., Shimak, R.M., Fong, R.H., Chafets, D., Bakkour, S., Keating, S., Fomin, M.E., Muench, M.O., Sherman, M.B., Doranz, B.J., Diamond, M.S., Simmons, G., 2015. Neutralizing monoclonal antibodies block chikungunya virus entry and release by targeting an epitope critical to viral pathogenesis. *Cell Rep.* 13, 2553–2564.
- Kuo, S.C., Chen, Y.J., Wang, Y.M., Kuo, M.D., Jinn, T.R., Chen, W.S., Chang, Y.C., Tung, K.L., Wu, T.Y., Lo, S.J., 2011. Cell-based analysis of chikungunya virus membrane fusion using baculovirus-expression vectors. *J. Virol. Methods* 175, 206–215.
- Lam, S., Chen, H., Chen, C.K., Min, N., Chu, J.J., 2015. Antiviral phosphorodiamidate morpholino oligomers are protective against chikungunya virus infection on cell-based and murine models. *Sci. Rep.* 5, 12727.
- Liu, K., Vergani, A., Zhao, P., Ben Nasr, M., Wu, X., Iken, K., Jiang, D., Su, X., Fotino, C., Fiorina, P., Visner, G.A., 2014. Inhibition of the purinergic pathway prolongs mouse lung allograft survival. *Am. J. Respir. Cell Mol. Biol.* 51, 300–310.
- Mancini, F., Toro, C.M., Mabilia, M., Giannangeli, M., Pinza, M., Milanese, C., 1999. Inhibition of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )/TNF- $\alpha$  receptor binding by structural analogues of suramin. *Biochem. Pharmacol.* 58, 851–859.
- Manimunda, S.P., Vijayachari, P., Uppoor, R., Sugunan, A.P., Singh, S.S., Rai, S.K., Sudeep, A.B., Muruganandam, N., Chaitanya, I.K., Guruprasad, D.R., 2010. Clinical progression of chikungunya fever during acute and chronic arthritic stages and the changes in joint morphology as revealed by imaging. *Trans. R. Soc. Trop. Med. Hyg.* 104, 392–399.
- Margolles-Clark, E., Jacques-Silva, M.C., Ganesan, L., Umland, O., Kenyon, N.S., Ricordi, C., Berggren, P.O., Buchwald, P., 2009. Suramin inhibits the CD40–CD154 costimulatory interaction: a possible mechanism for immunosuppressive effects. *Biochem. Pharmacol.* 77, 1236–1245.
- McGeary, R.P., Bennett, A.J., Tran, Q.B., Cosgrove, K.L., Ross, B.P., 2008. Suramin: clinical uses and structure-activity relationships. *Mini Rev. Med. Chem.* 8, 1384–1394.
- Miner, J.J., Aw Yeang, H.X., Fox, J.M., Taffner, S., Malkova, O.N., Oh, S.T., Kim, A.H., Diamond, M.S., Lenschow, D.J., Yokoyama, W.M., 2015. Chikungunya viral arthritis in the United States: a mimic of seronegative rheumatoid arthritis. *Arthritis Rheumatol.* 67, 1214–1220.
- Mishra, P., Kumar, A., Mamidi, P., Kumar, S., Basantray, I., Saswat, T., Das, I., Nayak, T.K., Chattopadhyay, S., Subudhi, B.B., Chattopadhyay, S., 2016. Inhibition of chikungunya virus replication by 1-[(2-methylbenzimidazol-1-yl) methyl]-2-oxo-indolin-3-ylidene] amino] thiourea(MBZM-N-IBT). *Sci. Rep.* 6, 20122.
- Morrison, T.E., Oko, L., Montgomery, S.A., Whitmore, A.C., Lotstein, A.R., Gunn, B.M., Elmore, S.A., Heise, M.T., 2011. A mouse model of chikungunya virus-induced musculoskeletal inflammatory disease: evidence of arthritis, tenosynovitis, myositis, and persistence. *Am. J. Pathol.* 178, 32–40.
- Mylonas, A.D., Brown, A.M., Carthew, T.L., McGrath, B., Purdie, D.M., Pandeya, N., Vecchio, P.C., Collins, L.G., Gardner, I.D., de Looze, F.J., Raymond, E.J., Suhrbier, A., 2002. Natural history of Ross River virus-induced epidemic polyarthritides. *Med. J. Aust.* 177, 356–360.
- Ozden, S., Huerre, M., Riviere, J.P., Coffey, L.L., Afonso, P.V., Mouly, V., de Monredon, J., Roger, J.C., El Amrani, M., Yvin, J.L., Jaffar, M.C., Frenkiel, M.P., Sourisseau, M., Schwartz, O., Butler-Browne, G., Despres, P., Gessain, A., Ceccaldi, P.E., 2007. Human muscle satellite cells as targets of chikungunya virus infection. *PLoS One* 2, e527.
- Pal, P., Dowd, K.A., Brien, J.D., Edeling, M.A., Gorlatov, S., Johnson, S., Lee, I., Akahata, W., Nabel, G.J., Richter, M.K., Smit, J.M., Fremont, D.H., Pierson, T.C., Heise, M.T., Diamond, M.S., 2013. Development of a highly protective combination monoclonal antibody therapy against chikungunya virus. *PLoS Pathog.* 9, e1003312.
- Piper, A., Ribeiro, M., Smith, K.M., Briggs, C.M., Huitt, E., Nanda, K., Spears, C.J., Quiles, M., Cullen, J., Thomas, M.E., Brown, D.T., Hernandez, R., 2013. Chikungunya virus host range E2 transmembrane deletion mutants induce protective immunity against challenge in C57BL/6J mice. *J. Virol.* 87, 6748–6757.
- Poo, Y.S., Nakaya, H., Gardner, J., Larcher, T., Schroder, W.A., Le, T.T., Major, L.D., Suhrbier, A., 2014a. CCR2 deficiency promotes exacerbated chronic erosive neutrophil-dominated chikungunya virus arthritis. *J. Virol.* 88, 6862–6872.
- Poo, Y.S., Rudd, P.A., Gardner, J., Wilson, J.A., Larcher, T., Colle, M.A., Le, T.T., Nakaya, H.I., Warrilow, D., Allcock, R., Bielefeldt-Ohmann, H., Schroder, W.A., Khromykh, A.A., Lopez, J.A., Suhrbier, A., 2014b. Multiple immune factors are involved in controlling acute and chronic chikungunya virus infection. *PLoS Negl. Trop. Dis.* 8, e3354.
- Ren, P., Zou, G., Bailly, B., Xu, S., Zeng, M., Chen, X., Shen, L., Zhang, Y., Guillon, P., Arenzana-Seisdedos, F., Buchy, P., Li, J., von Itzstein, M., Li, Q., Altmeyer, R., 2014. The approved pediatric drug suramin identified as a clinical candidate for the treatment of EV71 infection-suramin inhibits EV71 infection in vitro and in vivo. *Emerg. Microbes Infect.* 3, e62.
- Rodriguez-Morales, A.J., Cardona-Ospina, J.A., Villamil-Gomez, W., Paniz-Mondolfi, A.E., 2015. How many patients with post-chikungunya chronic inflammatory rheumatism can we expect in the new endemic areas of Latin America? *Rheumatol. Int.* 35, 2091–2094.
- Romas, E., Sims, N.A., Hards, D.K., Lindsay, M., Quinn, J.W., Ryan, P.F., Dunstan, C.R., Martin, T.J., Gillespie, M.T., 2002. Osteoprotegerin reduces osteoclast numbers and prevents bone erosion in collagen-induced arthritis. *Am. J. Pathol.* 161, 1419–1427.
- Rosario, V., Munoz-Louis, R., Valdez, T., Adames, S., Medrano, J., Paulino, I., Paula, J., Alba-Feriz, R., 2015. Chikungunya infection in the general population and in patients with rheumatoid arthritis on biological therapy. *Clin. Rheumatol.* 34, 1285–1287.
- Rothan, H.A., Bahrani, H., Abdulrahman, A.Y., Mohamed, Z., Teoh, T.C., Othman, S., Rashid, N.N., Rahman, N.A., Yusof, R., 2016. Mefenamic acid in combination with ribavirin shows significant effects in reducing chikungunya virus infection in vitro and in vivo. *Antivir. Res.* 127, 50–56.
- Rulli, N.E., Rolph, M.S., Srikiatkachorn, A., Anantapreecha, S., Guglielmotti, A., Mahalingam, S., 2011. Protection from arthritis and myositis in a mouse model of acute chikungunya virus disease by bandarit, an inhibitor of monocyte chemotactic protein-1 synthesis. *J. Infect. Dis.* 204, 1026–1030.
- Safadi, R.A., Musleh, A.S., Al-Khateeb, T.H., Hamasha, A.A., 2010. Analysis of immunohistochemical expression of k19 in oral epithelial dysplasia and oral squamous cell carcinoma using color deconvolution-image analysis method. *Head Neck Pathol.* 4, 282–289.
- Sahu, D., Saroha, A., Roy, S., Das, S., Srivastava, P.S., Das, H.R., 2012. Suramin ameliorates collagen induced arthritis. *Int. Immunopharmacol.* 12, 288–293.
- Schilte, C., Staikowsky, F., Couderc, T., Madec, Y., Carpentier, F., Kassab, S., Albert, M.L., Lecuit, M., Michault, A., 2013. Chikungunya virus-associated long-term arthralgia: a 36-month prospective longitudinal study. *PLoS Negl. Trop. Dis.* 7, e2137.
- Schuffenecker, I., Itean, I., Michault, A., Murri, S., Frangeul, L., Vaney, M.C., Lavenir, R., Pardigon, N., Reynes, J.M., Pettinelli, F., Biscornet, L., Diancourt, L., Michel, S., Duquerroy, S., Guigon, G., Frenkiel, M.P., Brehin, A.C., Cubito, N., Despres, P., Kunst, F., Rey, F.A., Zeller, H., Brisse, S., 2006. Genome microevolution of chikungunya viruses causing the Indian Ocean outbreak. *PLoS Med.* 3, e263.
- Suhrbier, A., La Linn, M., 2004. Clinical and pathologic aspects of arthritis due to Ross River virus and other alphaviruses. *Curr. Opin. Rheumatol.* 16, 374–379.
- Toivanen, A., 2008. Alphaviruses: an emerging cause of arthritis? *Curr. Opin. Rheumatol.* 20, 486–490.
- Tsetsarkin, K.A., Vanlandingham, D.L., McGee, C.E., Higgs, S., 2007. A single mutation in chikungunya virus affects vector specificity and epidemic potential. *PLoS Pathog.* 3, e201.
- Vazeille, M., Moutailler, S., Coudrier, D., Rousseaux, C., Khun, H., Huerre, M., Thiria, J., Dehecq, J.S., Fontenille, D., Schuffenecker, I., Despres, P., Failloux, A.B., 2007. Two chikungunya isolates from the outbreak of La Reunion (Indian Ocean) exhibit different patterns of infection in the mosquito, *Aedes albopictus*. *PLoS One* 2, e1168.
- Wang, D., Suhrbier, A., Penn-Nicholson, A., Woraratanadharm, J., Gardner, J., Luo, M., Le, T.T., Anraku, I., Sakalian, M., Einfeld, D., Dong, J.Y., 2011. A complex adenovirus vaccine against chikungunya virus provides complete protection against viraemia and arthritis. *Vaccine* 29, 2803–2809.
- Yoneda, T., Williams, P., Rhine, C., Boyce, B.F., Dunstan, C., Mundy, G.R., 1995. Suramin suppresses hypercalcemia and osteoclastic bone resorption in nude mice bearing a human squamous cancer. *Cancer Res.* 55, 1989–1993.
- Zeana, C., Kelly, P., Heredia, W., Cifuentes, A., Franchin, G., Purswani, M., Tieng, A., Haggmann, S.H., 2016. Post-chikungunya rheumatic disorders in travelers after return from the Caribbean. *Travel Med. Infect. Dis.* 14, 21–25.