Antiviral treatment efficiently inhibits chikungunya virus infection in the joints of mice during the acute but not during the chronic phase of the infection

Rana Abdelnabi¹, Dirk Jochmans¹, Erik Verbeken², Johan Neyts^{1#}, Leen Delang^{1#}*

1. University of Leuven, Department of Microbiology and Immunology, Rega Institute for Medical Research, Laboratory of Virology and Chemotherapy, B-3000 Leuven, Belgium.

2. University of Leuven and Leuven University Hospitals, Department of Pathology, B-3000 Leuven, Belgium.

#equal contribution

***Corresponding author:** Johan Neyts, Herestraat 49, 3000 Leuven, Belgium. Tel +3216321105, email: Johan.Neyts@kuleuven.be.

Abstract

Favipiravir (T-705) is a broad spectrum antiviral which has been approved in Japan for the treatment of severe influenza virus infections. We reported earlier that favipiravir inhibits the *in vitro* replication of CHIKV and protects against disease progression in CHIKV-infected immunodeficient mice. We here explored whether favipiravir is also able to inhibit CHIKV replication in the joints of mice either when treatment is initiated during the acute or during the chronic phase of the infection. To this end, C57BL/6J mice were infected with CHIKV in the left hind footpad and treatment with favipiravir (300 mg/kg/day, orally) was either given from day 0 to day 3 post-infection (p.i.) or from day 49 to day 55 p.i. In the untreated mice, viral RNA was still detectable in the joints up to 98 days p.i., yet no infectious viral particles were observed in these tissues. The 4 days treatment during the acute phase of the infection resulted in complete inhibition of systemic viral spread. As a consequence, no viral RNA was detected in the non-inoculated feet in contrast to the situation in the untreated control mice. When treatment was initiated at day 49 p.i., no significant reduction in viral RNA levels in joints were noted as compared to the untreated control. Interestingly, when attempting to amplify by RT-PCR material corresponding to virus genome from the chronic phase samples, some parts of the genome, such as the viral polymerase gene could not be amplified. Collectively, these results suggest that the viral RNA detected in the joints during the chronic phase is likely defective, which also explains the lack of effect of a viral replication inhibitor.

Key words:

Favipiravir; CHIKV; persistence; joints; mice

Body of text

Chikungunya virus (CHIKV) is a re-emerging arbovirus belonging to the genus *Alphavirus* of the family *Togaviridae* (Morrison et al., 2016). The symptoms of an acute CHIKV infection include fever, arthralgia, and, in many cases, maculopapular rash (Gasque et al., 2015). Although a CHIKV infection is rarely fatal, patients often severely suffer from chronic debilitating polyarthritis that can last for weeks to years after the acute infection (Gasque et al., 2015; Morrison et al., 2016). The exact mechanism by which CHIKV induces chronic arthritis has not been clarified yet. It was suggested that the virus may persist in synovial macrophages, thereby creating a reservoir and thus at day 2 post-infection (p.i.) escaping the immune response (Labadie et al., 2010; Singh and Unni, 2011). CHIKV antigens and RNA were isolated from the synovial tissues of a patient suffering from chronic pain 18 months after the CHIKV infection (Hoarau et al., 2010). Increased levels of inflammation markers (IFN- α , IL-6, MCP-1/CCL-2, IL-8, and MMP2) were also observed in the synovium of this patient (Hoarau et al., 2010). Similarly, CHIKV RNA remained detectable in musculoskeletal tissues of non-human primates (Labadie et al., 2010) for months after infection. A CHIKV-induced arthritis mouse model has been reported in which 2 to 3 weeks old C57BL/6J mice were inoculated with CHIKV in one of the hind footpads (Hawman et al., 2013; Morrison et al., 2011). The virus was shown to replicate efficiently in the joints (Morrison et al., 2011) and viral RNA was still detected in the joints of infected mice up to 16 weeks p.i. (Hawman et al., 2013). However, it is not clear whether the CHIKV RNA detected during the chronic stage in the joints results from active viral replication or is rather the consequence of delayed clearance of viral material. It also remains to be studied whether or not CHIKV inhibitors may have a beneficial effect on virus induced chronic infection and viral persistence.

Several studies reported the development of candidate CHIKV vaccines (Ahola et al., 2015; Schwameis et al., 2016) and the therapeutic potential of anti-CHIKV molecules *in vitro* and in animal models (Abdelnabi et al., 2015). However, there are today no vaccines nor antiviral drugs for the prevention or treatment of CHIKV infections. Exploring the potential efficacy of drugs that have already been approved for other diseases, as a treatment for CHIKV infections may therefore shorten the path towards developing such therapy. One of such drugs is favipiravir (T-705), which has been approved in Japan for the treatment of novel or re-emerging influenza virus infections that are not responding to treatment with other anti-influenza drugs (Furuta et al., 2013). Favipiravir is also endowed with antiviral activity against a broad range of viruses including, but not limited to, noroviruses (Rocha-Pereira et al., 2012), alphaviruses (Delang et al., 2014), flaviviruses (Zmurko et al., 2016), Rift Valley fever virus (Scharton et al., 2014), arenaviruses (Gowen et al., 2013) and paramyxoviruses (Jochmans et al., 2016). The potential antiviral efficacy of favipiravir has also been explored in Ebola virus-infected patients in Western Africa (Mentré et al., 2015).

Favipiravir is converted intracellularly into its ribose-5'-monophosphate (RMP) by the (human) hypoxanthine guanine phosphoribosyl transferase (HGPRT) followed by the formation of the ribose-5'-triphosphate metabolite (RTP). It has been shown, for the influenza virus, that favipiravir-RTP can be recognized as a substrate by the RNA-dependent RNA polymerase (RdRp) resulting in the inhibition of viral RNA synthesis (Furuta et al., 2013). However, the precise molecular mechanism of the antiviral activity of this compound has not yet been fully elucidated.

In an earlier study from our laboratory, favipiravir and its defluorinated analogue, T-1105, were shown to inhibit the *in vitro* replication of CHIKV (Delang et al., 2014). In a CHIKV-

infection model in AG129 mice, favipiravir treatment protected mice from severe neurological disease and reduced the number of mice to be euthanized by > 50% (Delang et al., 2014).

We here wanted to explore whether favipiravir is also able to inhibit CHIKV replication in the joints of infected C57BL/6J mice either when treatment is initiated during the acute phase of the infection or when the drug is first given during the chronic phase of the infection. The results of this study may thus help to provide an answer to the clinically very relevant question as to whether or not antivirals may result in a clinical benefit during the chronic stage of CHIKV infections in man.

To evaluate the potential protective effect of the broad-spectrum antiviral agent favipiravir during either the acute or chronic phase of a CHIKV infection, three weeks old C57BL/6J mice were injected with 10^3 PFU of CHIKV-899 strain (belongs to the Indian Ocean lineage, GenBank FJ959103.1) in the left footpad. Tissue samples were collected on days 0, 2, 4, 49, 56, and 98 p.i. for the detection of viral RNA levels and infectious virus titers. In both the left and right ankles, high levels of viral RNA were detected at day 2 and 4 p.i. and viral RNA remained detectable until 98 days p.i. (Supplementary Fig. S1A/B). The presence of infectious viral particles was assessed in the left hands and wrists and was readily detected at days 2 and 4 p.i. (Supplementary Fig. S1C). Interestingly however, infectious viral particles were no longer detected in the joints of hands and wrists of the infected animals at later time points despite the fact that viral RNA was readily detectable in other joints (Supplementary Fig. S1C).

To assess the potential antiviral effect of favipiravir during the acute phase of the CHIKV infection in this model, a group of mice were treated for 4 consecutive days with favipiravir at a dose of 300mg/kg/day divided over 2 daily dosing via oral gavage whereby the first dose was given immediately before infection. Serum and selected tissues were collected at days 2 and 4 p.i. and viral RNA and the infectious virus titers were determined. Treatment with favipiravir resulted in undetectable levels of viral RNA in serum (Fig. 1A), left lymph nodes (Fig. 1B) and right ankles (Fig. 1D) at day 4 p.i. In the ankles of the inoculated feet, 1.2 log₁₀ reduction in viral RNA was observed at day 4 p.i. (Fig. 1C). The reduction in viral RNA in both left and right ankles clearly indicates that favipiravir is distributed to and activated into its active ribose-5'-triphosphate form in the joints.

Titers of infectious virus were also determined in the spleen and the left hands and wrists of treated and untreated mice. No infectious virus particles were detected in the spleens (Fig. 2A) and left hands/wrists (Fig. 2B) of the animals in the treated group at day 2 and 4 p.i.

Immunostaining analysis on the left metatarsophalangeal tissues collected at day 4 p.i. revealed the accumulation of CHIKV antigens in the untreated mice (Supplementary Fig. S2A) whereas no viral antigens were detected in the tissues of the favipiravir-treated mice (Supplementary Fig. S2B) similar to the uninfected control (Supplementary Fig. S2C). It was next studied whether late start of treatment with favipiravir, i.e. at day 49 p.i. for 7 consecutive days (i.e. during the chronic phase of infection) can result in a reduction of viral RNA in the joints of infected mice. The left and right ankles were collected 1 week and 6 weeks after the end of treatment (i.e. days 56 and 98 p.i.). Strikingly, no significant reduction in viral RNA levels were observed in the treated animals (Fig. 3A/B).

The fact that favipiravir is not able to reduce CHIKV RNA in the joints during the chronic stage of infection may indicate that the detected viral RNA at this stage does no longer represent replicating virus but rather defective viral genomes or degraded RNA. To explore this hypothesis, the coding region of viral RNAs isolated from the original viral inoculum or from the left ankles at either day 2 or day 49 p.i. was amplified in the form of 7 complementary cDNA fragments by means of RT-PCR. The resulting RT-PCR products were visualized by gel electrophoresis (Fig. 4). For the chronic phase samples (day 49 p.i.), the viral RNA dependent RNA polymerase (RdRp, nsP4) amplicons were missing and the amplicons of the viral non-structural protein 1 (nsP1) [encoding guanine-7-methyltransferase and guanylyl-transferase which are required for capping of the viral RNA] and envelope glycoprotein (E2) had a smaller size than expected (Fig. 4). On the other hand, the bands for amplicons of the day 2 samples were similar to those of the original inoculum except for the fact that an extra smaller size band was detected in the nsP1 amplicon sample (similar to the band observed in the day 49 p.i. samples) (Fig. 4). Sanger sequencing of the small E2 and nsP1 amplicons obtained from the day 49 p.i. samples revealed that the sequence of the E2 amplicon covers only a part of the CHIKV E2 gene. However, the sequence of the small amplicon in the nsP1 sample was not aligned to the CHIKV genome. Instead, it was representing a part of the nucleotide sequence of a mouse sodium channel (off target amplification). This explains the fact that a similar band was also observed during amplification of nsP1 gene from day 2 p.i. samples (Fig. 4).

These findings, together with the lack of antiviral efficacy of favipiravir in late stage infections, suggest that the viral RNA detected during the chronic phase of infection represents likely defective viral genomes that persists long after the replicating virus has

been cured by the immune system. However, the precise nature of the viral RNA detected during the chronic phase and its role in the pathogenesis of the chronic CHIKV-induced arthritis requires further study. If the observations made in the mouse model can be translated to the human situation, this would mean that antivirals (once available) will have to be used during the acute stage of the infection. This should reduce the severity of the disease during this phase and may also reduce the likelihood of the development of a chronic infection.

In conclusion, we here demonstrate for the first time that an inhibitor of CHIKV replication, such as favipiravir, is able to inhibit CHIKV replication in the joints of the extremities of infected mice (and other locations). Yet, when treatment is initiated several weeks after infection, when only viral RNA but not infectious virus is detected in joints, the antiviral effect is no longer observed.

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Conflict of interest

None to declare

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Zika Virus Replication and Delays Disease Progression in a Robust Mouse Infection

Model. PLoS Negl. Trop. Dis. 10. doi:10.1371/journal.pntd.0004695

Figures

Fig. 1. Effect of favipiravir treatment on viral RNA levels in ankles, lymph nodes and serum during the acute phase of CHIKV infection. Three weeks old C57BL/6J mice were injected with 10^3 PFU of CHIKV-899 strain in the left footpad. Favipiravir treatment (300 mg/kg/day, dosed twice daily via oral gavage) was initiated immediately before infection and was continued for the next 3 days. The sera and tissues from treated and untreated mice were collected on day 2 and 4 post-infection for quantification of viral RNA loads. Statistical analysis was performed using the unpaired, two-tailed t-test: ns, $p \geq 0.05$.

Fig. 2. Effect of favipiravir treatment on infectious virus titers in spleen and hand/wrist during the acute phase of CHIKV infection. Three weeks old C57BL/6J mice were inoculated with 10^3 PFU of CHIKV-899 strain in the left footpad. Favipiravir treatment (300mg/kg/day, dosed twice daily via oral gavage) was initiated immediately before infection and was continued for the next 3 days. Spleens and left hands/wrists from treated and untreated mice were collected on day 2 and 4 post-infection for quantification of infectious virus load. Statistical analysis was performed using the unpaired, two-tailed t-test.

Fig. 3. Efficacy of favipiravir treatment (from day 49 to day 55 p.i.) on viral RNA levels during the chronic phase of CHIKV infection. Three weeks old C57BL/6J mice were injected with 10^3 PFU of CHIKV-899 strain in the left footpad. Mice were treated with favipiravir (300mg/kg/day, dosed twice daily via oral gavage) from day 49 to day 55 p.i. (7 consecutive days). The ankles from treated and untreated mice were collected at days 56 and 98 p.i. and viral RNA loads were quantified. Statistical analysis was performed using the unpaired, two-tailed t-test: ns, $p \geq 0.05$.

Fig. 4. RT-PCR amplification of the coding region of CHIKV RNA isolated from infected mice. RNA extracted from either the viral inoculum or from left ankles of untreated infected mice was used to generate seven complementary cDNA fragments that represent the major part of CHIKV coding region. The RT-PCR products were separated and visualized by gel electrophoresis. The gel images are representative for 3 independent RT-PCR reactions (nsP=non-structural protein, E=envelope glycoprotein).

Supplementary figure legends:

Fig.S1. CHIKV RNA and infectious virus titers in the ankles and wrist during the acute and the chronic phase of the infection. Three week old C57BL/6J mice were inoculated with 10^3 PFU of CHIKV-899 strain in the left footpad. Samples were collected at the indicated time points. Viral RNA loads were determined in the left ankles (A) as well as the right ankles (B) and the infectious viral titers were determined in the left hands and wrists (C). The following primers were used for the quantitative RT-PCR: ChikSII 5'-CCGACTCAACCATCCTGGAT-3' and ChikAsII 5'-GGCAGACGCAGTGGTACTTCCT-3'.

Fig. S2. CHIKV antigens are not detectable in the joints of favipiravir-treated mice during the acute phase of CHIKV infection. Sections of the left metatarsophalangeal tissues of untreated and treated mice were collected at day 4 post-infection and stained with anti-CHIKV nsP1 antibody to detect viral antigens (dark brown staining). A) Untreated, B) favipiravir-treated and C) negative control (uninfected, untreated). Images are representative of the result from 3 different mice (magnification x400).