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Favipiravir inhibits *in vitro* Usutu virus replication and delays disease progression in an infection model in mice

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ABSTRACT

Usutu virus (USUV) is an emerging flavivirus that causes Usutu disease mainly in birds, but infection of mammals such as rodents, bats and horses has also been demonstrated. In addition, human cases (both in immunocompromised and -competent individuals) were also reported. Large outbreaks with other flaviviruses, such as West Nile virus and Zika virus, indicate that one should be vigilant for yet other outbreaks. To allow the identification of inhibitors of USUV replication, we established *in vitro* antiviral assays, which were validated using a small selection of known flavivirus inhibitors, including the broad-spectrum viral RNA polymerase inhibitor favipiravir (T-705). Next, an USUV infection model in AG129 (IFN-α/β and IFN-γ receptor knockout) mice was established. AG129 mice proved highly susceptible to USUV; an inoculum as low as 10^2 PFU (1.3×10^5 $TCID_{50}$) resulted in the development of symptoms as early as 3 days post infection with viral RNA being detectable in various tissues. Treatment of mice with favipiravir (150 mg/kg/dose, BID, oral gavage) significantly reduced viral load in blood and tissues and significantly delayed virus-induced disease. This USUV mouse model is thus amenable for assessing the potential *in vivo* efficacy of (novel) USUV/flavivirus inhibitors.

1. Main text

Usutu virus (USUV) is a mosquito-borne flavivirus originating from Africa, where it was first detected in the late 1950's. This virus is a relatively unknown member of the Japanese encephalitis serocomplex and is thus closely related to human pathogens such as Japanese encephalitis virus (JEV), yellow fever virus (YFV), Saint Louis encephalitis virus (SLEV), West Nile virus (WNV) and Murray Valley encephalitis virus (MVEV) (Moureau et al., 2015). The *Culex* mosquitoes are the primary vectors although other mosquito species such as *Aedes albopictus* have also been proposed as potential vectors (Eiden et al., 2018; Calzolari et al., 2010; Vázquez et al., 2011). USUV most likely emerged for the first time in Europe in 1996 causing massive deaths among blackbirds (*Turdus merula*) (Weissenböck et al., 2013). Currently, the virus is ubiquitous in Europe as its presence has been demonstrated in various animal species (e.g. birds, rodents, bats and horses) in more than ten European countries (Becker et al., 2012; Busquets et al., 2008; Cadar et al., 2014; Calzolari et al., 2012; Chvala et al., 2007; Garigliany et al., 2017; Hubálek et al., 2008; Lecollinet et al., 2016; Rijks et al., 2016; Steinmetz et al., 2011). Potentially even more concerning is the presence of USUV in humans. Seroprevalence studies showed that antibodies against USUV could be detected in the blood of 0.02% and 0.23%–1.1% of otherwise healthy blood donors in respectively Germany and Italy (Allering et al., 2012; Gaibani et al., 2012; Pierro et al., 2013). However, a higher seroprevalence was detected in serum samples from both healthy and sick individuals in Italy (6.6% seroprevalence) and healthy individuals in Serbia (7.5% seroprevalence), respectively (Cvjetković et al., 2016; Gaibani and Rossini, 2017; Grottola et al., 2017). USUV infections in humans remain largely asymptomatic. However, as of 2009, the number of USUV infections that are associated with disease, ranging from mild (rash, fever, headache) to severe disease (encephalitis, meningoencephalitis, meningitis), is slowly increasing in Europe. Although the first cases were reported on the African continent, more recent cases have been reported in Italy, Croatia, Germany, and France (Allering et al., 2012; Cavrini et al., 2009, 2011; Grottola et al., 2017; Nikolay et al., 2011; Pecorari et al., 2009; Simonin et al., 2018; Vilibic-Cavlek et al., 2014). The widespread distribution in different hosts in Europe provides evidence that the virus can jump between species and adapt to a new host. Hence, USUV should be considered a potential health concern. Given the recent -and

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mostly unexpected-outbreaks of related flaviviruses such as WNV, YFV, dengue virus and Zika virus (Musso et al., 2018; Weaver, 2018), it will thus be important to have broad-spectrum antivirals at hand that are also active against USUV.

To allow the identification of molecules that can inhibit *in vitro* USUV replication, antiviral assays were established. First, various cell types were tested for their permissiveness to USUV replication: Vero E6 (African Green monkey kidney) cells, BHK (baby hamster kidney) cells and C6/36 mosquito (from *Ae. albopictus* larvae) cells. Vero cells were highly permissive to USUV (strain V18; kindly provided by Dr. Stephan Günther, Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany; Becker et al., 2012), resulting in a full cytopathic effect (CPE) at day 7 post infection (pi) (data not shown). Hence, prior to performing subsequent experiments, USUV was propagated to a maximum of two passages in Vero E6 cells after which infectious titers were determined by performing plaque assays on BHK cells, essentially as described previously (Zmurko et al., 2016). The *in vitro* kinetics of USUV replication is comparable with that of other flaviviruses (Mastrangelo et al., 2012; Zmurko et al., 2016), with USUV RNA becoming first detectable by RT-qPCR at 6–8 h pi (Supplemental Fig. 1, grey bars). Next, a CPE-based assay followed by colorimetric read out (i.e. MTS/PMS; Promega) as well as a virus yield reduction assay followed by RT-qPCR was established using Vero E6 cells. Both assays were then validated using a selection of molecules with known antiviral activity against other flaviviruses (reviewed in Boldescu et al., 2017). All compounds were able to inhibit CPE formation as well as USUV RNA replication, with favipiravir as well as the 2′-*C*-methyl nucleoside analogs 2′-C-methyladenosine (2′CMA) and 7-deaza-2′-C-methyladenosine (7DMA) being the best, albeit not very potent, inhibitors, as based on the selectivity index (i.e. ratio CC_{50}/EC_{50}) (Table 1). More in detail, favipiravir was most effective in inhibiting USUV replication when added to the infected cells during the first 6 h after infection (Supplemental Fig. 1, black bars). The antiviral activity was lost when the compound was added after the onset of viral RNA synthesis, which corroborates with its proposed mechanism of action against other RNA viruses, i.e. acting as a mimetic nucleobase by interfering with the viral RNA synthesis (Delang et al., 2014; Furuta et al., 2005). The *in vitro* assays that were established are thus capable of identifying inhibitors of USUV replication.

To evaluate the *in vivo* efficacy of molecules against USUV, we aimed to establish a small animal model. Since USUV is an avian (flavi-) virus, we anticipated that the virus would replicate well in embryonated chicken eggs. USUV (10^4, 10^5 or 10^6 PFU) was injected in the allantoic cavity of chicken eggs ten days after fertilization, after which the allantoic fluid was harvested at various days (d2, d4 and d6) pi to determine infectious virus content. Surprisingly, only low USUV titers (i.e. 10^3 PFU/ml) were detected in only 14% of the chicken eggs, which may represent remnants of the input virus. In addition, all embryonated chicks developed normally (data not shown). Embryonated chicken eggs are thus not suitable to serve as a replication model for USUV.

Table 1
Antiviral activity of a selection of compounds against USUV and their anti-metabolic effect.

Compound	EC_{50} (μM)		CC_{50} (μM)
	CPE	RT-qPCR	
2′CMA	7.2 ± 0.1	12 ± 7.7	69 ± 1.3
2′CMC	13 ± 1.0	24 ± 9.1	71 ± 14
2′CMG	26 ± 0.8	43 ± 7.5	> 168
7DMA	45 ± 7.1	130 ± 2.1	> 357
Favipiravir	90 ± 26	151 ± 28	> 637
Ribavirin	139 ± 30	*ND*	> 410

Antiviral activities were determined in CPE reduction assays (CPE) followed by MTS/PMS read out or in virus yield reduction assay followed by real time quantitative PCR (RT-qPCR). Anti-metabolic activities were determined in toxicity assays followed by MTS read out. Data represent mean values ± standard deviations from at least three independent experiments with three replicates for each experiment (n = 9). 2′-C-methyladenosine, 2′CMA; 2′-*C*-methylcytidine, 2′CMC; 2′-*C*-methylguanosine, 2′CMG; 7-deaza-2′-C-methyladenosine, 7DMA. *ND*, not determined.

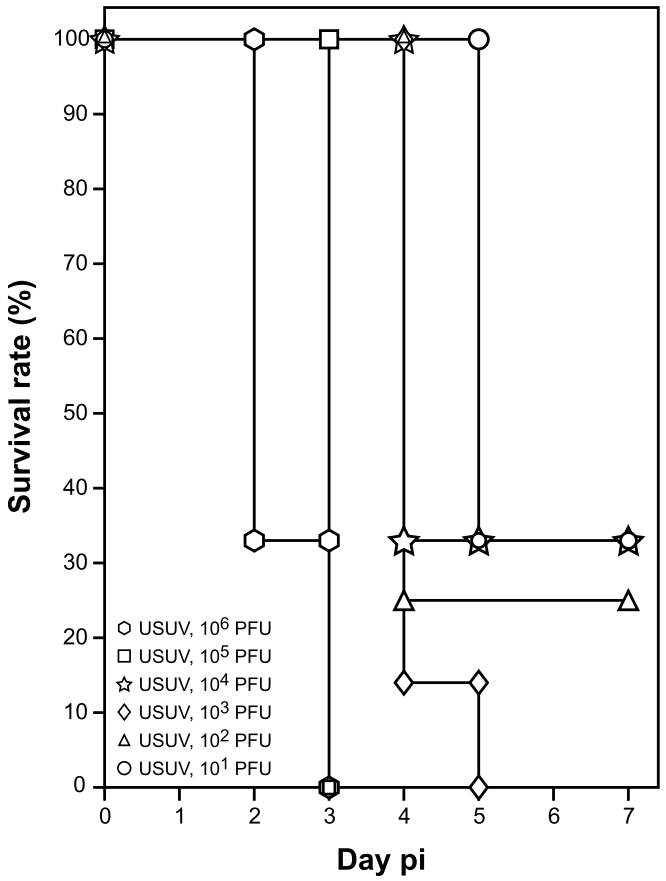

Fig. 1. Kaplan-Meier survival curve for mice infected with different inoculums of USUV. Male (8–14 weeks of age) 129/Sv mice deficient in both IFN-alpha/beta (IFN-α/β) and IFN-gamma (IFN-γ) receptors (AG129) were inoculated intraperitoneally (ip) with USUV V18 using different inoculums (ranging from 10^1 PFU - 10^6 PFU, corresponding to 1.3×10^4 $TCID_{50}$ - 1.3×10^9 $TCID_{50}$). Mice were observed daily for body weight loss and the development of virus-induced disease and were euthanized when reaching humane endpoints. In case of a body weight loss of ≥20% and/or severe illness (neurotropic symptoms such as movement impairment, lower limb paralysis), mice were euthanized with pentobarbital (Dolethal). All animal experiments were performed with approval of and under the guidelines of the Ethical Committee of KU Leuven [P099-2013].

We next studied whether AG129 mice can be infected with USUV. AG129 mice are deficient in IFN-α/β and IFN-γ receptors, which makes them highly susceptible to other (flavi-)viruses (Couderc et al., 2008; Rocha-Pereira et al., 2013; Schul et al., 2007; Zmurko et al., 2016), Moreover, it was previously reported that an USUV infection is partly regulated by the interferon response (Blázquez et al., 2015; Cacciotti et al., 2015; Martín-Acebes et al., 2016; Scagnolari et al., 2013), which is impaired in AG129 mice. Indeed, intraperitoneal (ip) infection of AG129 mice (8–14 weeks old) with USUV resulted in a rapid development of disease requiring euthanasia in the majority of the mice within a few days after infection (Fig. 1). When inoculated with 10^6 PFU (corresponding to 1.3×10^9 $TCID_{50}$), mice developed the first signs of disease 2 days pi and all mice had to be euthanized by day 3 pi. At the lowest inoculum used (10^1 PFU/1.3×10^4 $TCID_{50}$), first signs of disease appeared a few days later (day 4 pi) and most of the mice had to be euthanized by day 7 pi (33% survival). Disease signs include general

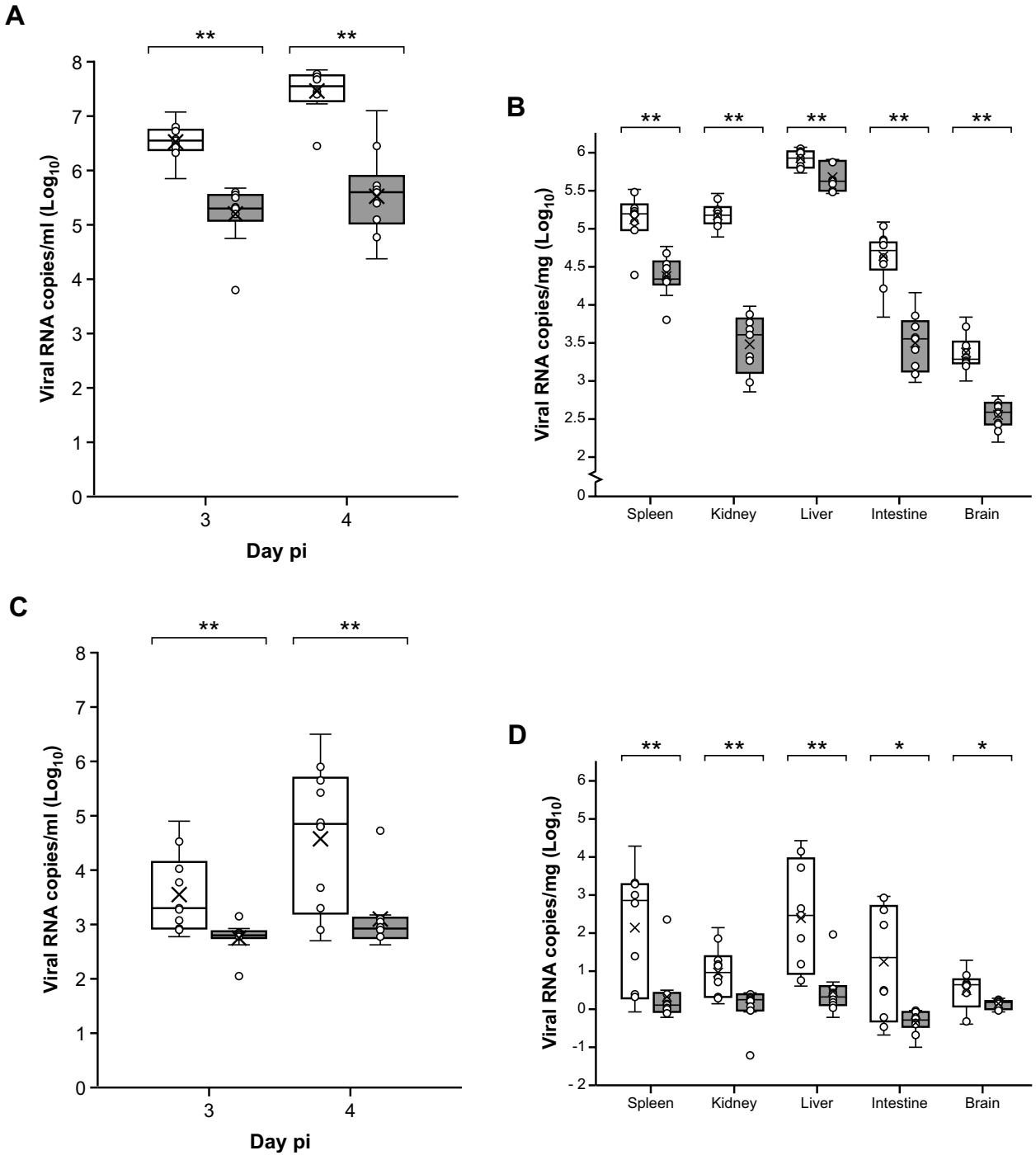

Fig. 2. Significant reduction in viral RNA load in serum and tissues of USUV-infected mice treated with favipiravir compared to those treated with vehicle. Male (8–14 weeks old) AG129 mice (n = 10/group) were inoculated ip with either 10^2 PFU/1.3 × 10^5 $TCID_{50}$ (**A** and **B**) or 10^0 PFU/1.3 × 10^3 $TCID_{50}$ (**C** and **D**) of USUV V18. Mice were treated twice-daily by oral gavage with favipiravir (T-705, 150 mg/kg/dose; grey bars) or vehicle (0.4% sodium carboxymethylcellulose, CMC-Na; white bars) for 5 consecutive days, starting one day prior to infection. Viral RNA load in serum (**A** and **C**) was determined on d3 and d4 pi, whereas viral RNA load in tissues (**B** and **D**) was determined at d4 pi, a time point at which all mice were sacrificed. Viral RNA was extracted from tissues using the RNeasy Mini Kit (Qiagen, The Netherlands) followed by viral RNA quantification by means of RT-qPCR. Statistical analysis was performed using the Shapiro–Wilk test for normality followed by the one-tailed Mann-Whitney *U* test. **, $p < 0.01$; *, $p < 0.05$ (Statistical tools, available from http://sdittami.altervista.org/shapirotest/ShapiroTest.html and http://in-silico.online, accessed May 2018).

symptoms (such as rapid body weight loss, hunched posture, and lethargy), conjunctivitis as well as neurotropic symptoms (such as movement impairment, lower limb paralysis). Next, viral RNA levels were monitored in the blood at d3-d5 pi to determine the day of peak viremia. In mice infected with either 10^2 PFU (1.3 × 10^5 $TCID_{50}$) or 10^3 PFU (1.3 × 10^6 $TCID_{50}$), comparable high levels of viral RNA were detected in the blood of infected mice at day 3 pi: 6.6 log_{10} and 6.5 log_{10}, respectively (Supplemental Fig. 2). Viral RNA levels increased further in the following days, reaching a peak level of 8.3log_{10} at d4 pi and 8.8log_{10} at d5 pi for mice infected with 10^2 PFU (1.3 × 10^5 $TCID_{50}$) and 10^3 PFU (1.3 × 10^6 $TCID_{50}$), respectively. At d5, pi the majority of the mice infected with the higher inoculum were euthanized, whereas

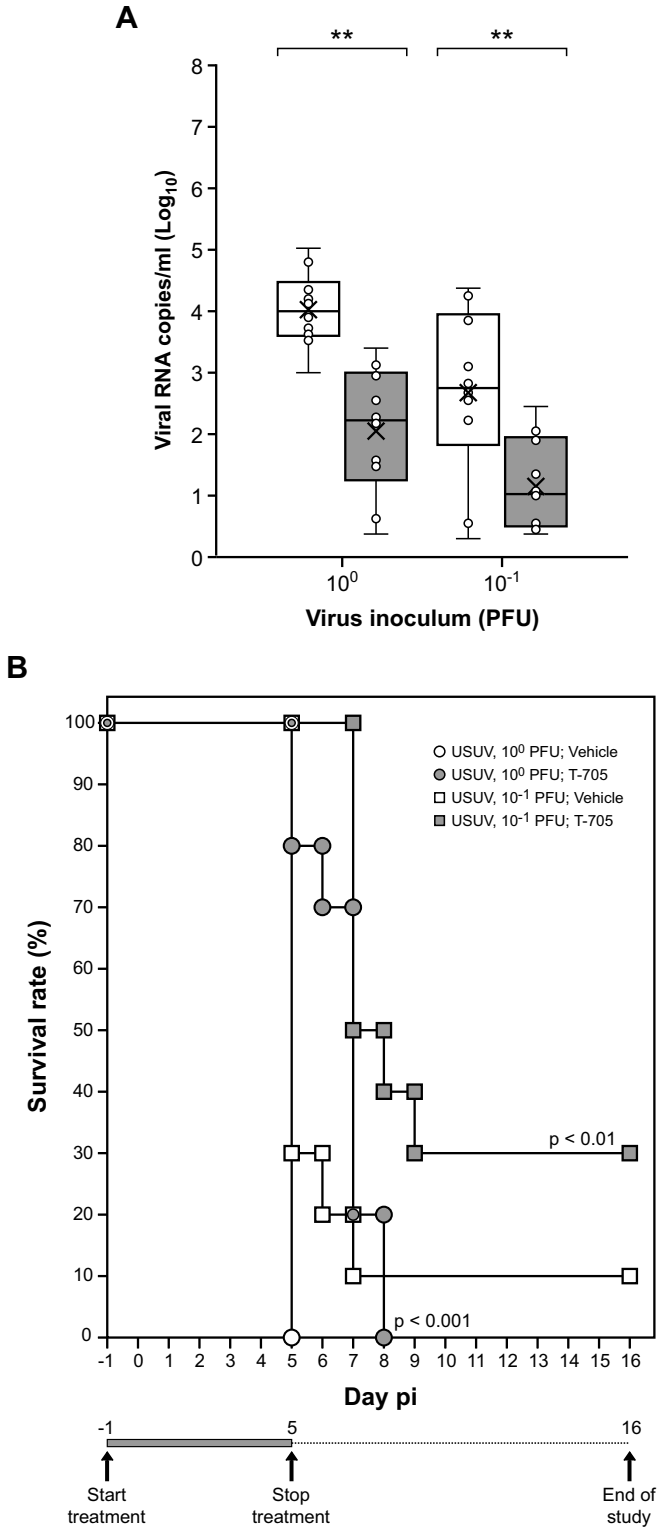

Fig. 3. Significant reduction in viremia (**A**) translates in significant delay in USUV-induced disease (**B**) in mice treated with favipiravir. Male (8–14 weeks old) AG129 mice (n = 10/group) were inoculated ip with either 10^0 PFU/1.3×10^3 $TCID_{50}$ (depicted by circles in **B**) or 10^{-1} PFU/1.3×10^2 $TCID_{50}$ (depicted by squares in **B**) of USUV V18. Mice were treated twice-daily by oral gavage with favipiravir (T-705, 150 mg/kg/dose; grey bars, squares or circles) or vehicle (0.4% CMC-Na; white bars, squares or circles) for 6 consecutive days, starting one day prior to infection. Mice were observed daily for body weight loss and the development of virus-induced disease. In case of a body weight loss of ≥20% and/or severe illness, mice were euthanized with pentobarbital (Dolethal). Viral RNA load in serum was determined at the day of peak viremia (d4 pi) by RT-qPCR. Statistical analysis was performed using the Shapiro–Wilk test for normality followed by the one-tailed Mann-Whitney *U* test (viremia) or the Log rank test (survival). **, $p < 0.01$ (Statistical tools, available from http://sdittami.altervista.org/shapirotest/ShapiroTest.html and http://in-silico.online, accessed May 2018).

this was the case for only one mouse in the lower inoculum group. Based on these findings, it was decided to quantify viremia in blood at d3 and d4 pi and in tissues at d4 pi in all further experiments using an inoculum of 10^2 PFU (1.3×10^5 $TCID_{50}$) or lower.

The USUV mouse model was then validated for suitability in antiviral studies. To this end, favipiravir was used, which was earlier shown to be active in mouse infection models with other (related) viruses (Delang et al., 2014; Furuta et al., 2002; Gowen et al., 2007; Julander et al., 2009; Morrey et al., 2008; Rocha-Pereira et al., 2012). Treatment of mice infected with 10^2 PFU (1.3×10^5 $TCID_{50}$) USUV for 5 consecutive days with favipiravir (150 mg/kg/dose, BID, oral gavage) resulted in a statistically significant reduction of viral RNA levels in the blood as compared to vehicle-treated mice at both d3 and d4 pi: $1.3\log_{10}$ and 1.9 $\log_{10}$, respectively (Fig. 2A). In addition, viral RNA levels were also statistically significantly reduced in tissues (spleen, kidney, liver, intestine and brain) as compared to vehicle-treated mice (Fig. 2B). The reduction in viremia appeared to be independent on the inoculum used, as significant reductions in viral RNA load were also observed in favipiravir-treated mice infected with a 100 fold lower virus inoculum (i.e. 10^0 PFU/1.3×10^3 $TCID_{50}$) (Fig. 2C and D).

Finally, it was assessed whether the reduction in viremia that is brought about by favipiravir translates into a significant delay of virus-induced disease progression. Mice were infected with 10^0 PFU (1.3×10^3 $TCID_{50}$; as in Fig. 2C and D) or a lower inoculum (i.e., 10^{-1} PFU/1.3×10^2 $TCID_{50}$). Treatment with favipiravir significantly reduced viremia by $1.8\log_{10}$ and 1.7 $\log_{10}$ in mice infected with 10^0 PFU respectively 10^{-1} PFU (Fig. 3A), as was shown before. In addition, treatment with the compound significantly delayed virus-induced disease in both groups (Fig. 3B). In mice infected with 10^0 PFU, the mean day of euthanasia (MDE) was 5.0 and 6.7 for mice treated with vehicle or favipiravir, respectively, whereas the MDE was 6.4 (vehicle) and 10 (favipiravir) in mice infected with a 10-fold lower inoculum. Favipiravir could not halt the progression of the disease and ultimately all 10 mice (inoculated with 10^0 PFU/1.3×10^3 $TCID_{50}$), respectively 7 out 10 mice (inoculated with 10^{-1} PFU/1.3×10^2 $TCID_{50}$), had to be euthanized.

In conclusion, the USUV infection model in AG129 mice represents a suitable model for use in antiviral studies. USUV causes a particularly fulminant infection in AG129 mice, which may also explain the rather modest protection that is achieved by the reference compound favipiravir.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://

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